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13. ABSTRACT (Maximum 200 Words)

Our **Graduate Training Program in Breast Cancer Biology and Therapy** is a multidisciplinary approach focused on an important disease. The overall philosophy of our training program is to identify qualified graduate students in the existing discipline-based training programs and to interest and educate them in the unsolved problems in breast cancer.

The specific goals of our Program are: **a)** To recruit qualified predoctoral students to breast cancer related research; **b)** To educate students in the fundamental principles of breast cancer pathobiology and therapy; **c)** To monitor and evaluate the progress of the enrolled students and mentor them in their future career choices; **d)** To organize program activities, such as Seminar Series and Journal Clubs, for increased interaction of the student trainees with postdoctoral fellows and faculty interested in breast cancer.

We have completed the last year of the training program in which we have closely followed our specific goals. Six students were trained. They published 4 manuscripts, three chapters and 20 abstracts on their research related to breast cancer.

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INTRODUCTION

Our **Graduate Training Program in Breast Cancer Biology and Therapy** has had as a multidisciplinary approach focused on an important disease. The overall philosophy of our training program has been to identify qualified graduate students in the existing discipline-based training programs and to interest and educate them in the unsolved problems in breast cancer. By raising their interest and providing them with financial help, we encouraged them to apply the tools of their individual disciplines in search of solutions to breast cancer-related problems. Our Training Program has expanded the existing pool of investigators studying breast cancer. The Program also encouraged many of our faculty members, by including them into the list of Program Faculty and by providing support for their graduate students, to focus their research effort at least in part on breast cancer. This has been an important programmatic by-product because it has taken ongoing interdisciplinary research effort by an array of well-funded investigators and directed it towards the problems of breast cancer. The specific goals of our Program were: **a**)To recruit qualified predoctoral students to breast cancer related research. **b**)To educate students in the fundamental principles of breast cancer pathobiology and therapy. **c**)To monitor and evaluate the progress of the enrolled students and mentor them in their future career**d**)choices.To organize program activities, such as Seminar Series and Journal Clubs, for increased interaction of the student trainees with postdoctoral fellows and faculty interested in breast cancer. We have completed the fourth and last year of the training program in which we have closely followed our specific goals. This four-year grant was the second we received and in the total of eight years funded by the two consecutive grants, we have trained 24 graduate students. We are very disappointed that this funding mechanism has been eliminated in favor of individual training grants. The students trained under this institutional training grant mechanism receive much more intellectual support from a team effort that keeps the focused and promotes research collaborations.

BODY (ANNUAL SUMMARY)

Progress in Trainee Recruitment: In this last year of the grant we completed training of six students who were selected for funding in the previous year. This last group of trainees was appointed in 2001 through our standard nomination, competition and selection process. All training faculty, as well as the community at large, received an electronically transmitted letter informing them of the program and requesting trainee nominations. Applications were submitted also electronically and evaluated by the **Breast Cancer Training Grant Executive Committee**. The committee members were Drs. Finn, Lazo, Morris and Latimer. Applicants were judged based on the student's performance in the first and second year of graduate school, faculty comments, and a brief written statement of their research interest as related to breast cancer. An effort was made to ensure equitable distribution of fellowships among multiple disciplines and areas of research. Five new students were selected to receive funding, four starting September 1, 2001 and the fifth January 1, 2002. In August 2002, one of our students graduated early and we awarded one-year fellowship to a new student whose work will be described below.

Progress in Trainee Education and Monitoring of Progress: Inasmuch as the students supported by this training grant belong to various graduate programs, the formal course work requirements and credits of dissertation research are determined by their individual programs. **The Training Program in Breast Cancer Biology and Therapy** requires that the students complete an Ethics course offered by the University and attend the weekly conference on Breast Cancer Biology and Therapy organized every Thursday afternoon by Dr. Jean Latimer. Each student is required to present a seminar in this series at least once during the two year period of support under the training grant..The Program Director, Dr. Finn, monitors all seminars campus wide and alerts the trainees to those of special interest to breast cancer.

Progress in Organizing Program Activities: In addition to several established seminar series that our trainees attend, the Magee Research Institute of the University of Pittsburgh and the University of Pittsburgh Cancer Institute Breast Program organized a series of monthly Debates on Breast Cancer Care, which has now been named Seminars on Women's Health. Attendance is required of all the trainees.

Research Accomplishments of individual trainees from 9/1/2002 until 8/31/2003

Zoya Shurin (Dr. Paul Robbins, advisor), Bioengineering Graduate Program. Zoya has studied the escape from immune surveillance as a fundamental feature of tumors, which contributes to their uncontrolled growth. The escape of malignant cells from immune recognition results from a defective function of cells of the immune system, including DC. CD40 plays an important role in both antitumor immunity and DC maturation. The interaction between CD40 on antigen-presenting cells and its ligand CD40L (CD154) on T cells plays an

important role in the induction of immune responses, including anti-tumor immunity. Soluble CD40L and transfer of the CD40L gene to tumor cells have been shown to induce specific immune responses in several murine tumor models. In her studies to date, Zoya, has evaluated whether expression of CD40L at the site of the tumor elicits an immune response to established tumors in mice. A recombinant adenovirus encoding murine CD40L (Ad-CD40L) was constructed and tested in the TS/A breast adenocarcinoma model. Administration of Ad-CD40L on Day 7 after tumor inoculation resulted in a significant inhibition of tumor growth when compared with the control groups treated with either saline or control adenovirus. She has examined the therapeutic efficacy of intratumoral injection of murine bone marrow-derived dendritic cells (DC) transduced with adenovirus encoding the CD40L. Intratumoral injection of DC/CD40L resulted in the majority of the animals being tumor-free 20 days post-therapy which was associated with induction of systemic immunity. Moreover, she has demonstrated that DC overexpressing CD40L have no direct effect on TS/A breast adenocarcinoma cells in vitro, suggesting that DC/CD40L induced a specific antitumor response in vivo. Interestingly, DC/CD40L produced significantly higher levels of IL-12 than control DC, suggesting additional pathways of the antitumor activity of DC/CD40L. Her data demonstrate that Ad-CD40L transduction of DC or tumor cells at the site of the tumor may be an effective approach to induce an antitumor immune response to breast cancer.

Publications:

1. Shurin M.R., **Yurkovetsky Z.R.**, Barksdale E. Jr., Shurin G.V. Inhibition of CD40 expression during dendropoiesis by tumor: Role of GM3 and IL-10. Submitted.
2. Tourkova I.L., **Yurkovetsky Z.R.**, Gambotto A., Shurin M.R., Shurin G.V. Increased Function and Survival of IL-15-transfected Human Dendritic Cells are Mediated by Up-regulation of IL-15Rα and Bcl-2. Submitted.
3. Satoh Y., Esche C., Gambotto A., Shurin G.V., **Yurkovetsky Z.R.**, Robbins P.D., Watkins S.C., Todo S., Lotze M.T., Herberman R.B., Shurin M.R. Local Administration of IL-12-transfected Dendritic Cells Induces Antitumor Immune Responses to Colon Adenocarcinoma in the Liver in Mice. *J. Exp. Therap. Oncol.* In press.
4. Yamabe K., Peron J.M., Esche C., **Yurkovetsky Z.R.**, Watkins S., Lotze M.T., Shurin M.R. Lymphoid and myeloid dendritic cells: Functional differences between in vivo and in vitro generated cells. Submitted.
5. **Yurkovetsky, Z.R.**, Shurin G.V., Gambotto A., Kim S.H., Shurin M.R., Robbins P.D. Intramumoral administration of adenovectors encoding the CD40L gene dendritic cells transduced with CD40L vector induces antitumor immunity in mice. Cancer Gene Therapy. Submitted

Abstracts:

1. **Yurkovetsky Z.R**, Gambotto A., Kim S.H., Shurin M.R., Robbins P.D. Intratumoral administration of Ad-CD40L or DC/CD40L elicited effective antitumor immunity in mice. 13th annual Virology Symposium, 2002.
2. **Yurkovetsky Z.R.,** Robbins P.D. Antitumor effect of adenoviral vectors expressing CD40L, RANKL or 4-1BBL. ASGT 5th annual meeting, 2002.
3. Kin S.H., **Yurkovetsky Z.R.,** Robbins P.D. Combined immunotherapy of a murine mammary tumor using genetically modified dendritic cells. ASGT 5th annual meeting, 2002.
4. **Yurkovetsky Z.R.,** Robbins P.D. Antitumor effect of adenoviral vectors expressing CD40L, RANKL or 4-1BBL. MGB retreat, 2002.
5. **Yurkovetsky Z.R.**, Shurin M.R., Robbins P.D. Intratumoral administration of adeno CD40L or dendritic cells overexpressing CD40L elicited effective antitumor immunity in mice. Era of Hope, DOD breast cancer research program meeting, 2002
6. Pirtskhalaishvili G., Gambotto A., Esche C., **Yurkovetsky Z.R.**, Lotze M.T., Shurin M.R. IL-12 and Bcl-xl gene transfection of murine dendritic cells protects them from prostate cancer-induced apoptosis and improves their antitumor activity. J. Urol. 163 (Suppl): 105, 2000.

7. Tourkova I., Shurin G.V., Gambotto A., **Yurkovetsky Z.R.**, Shurin M.R. Transfection of dendritic cells with IL-15 gene protects them from prostate cancer induced apoptosis. Keystone Meeting "Dendritic cells'. Taos, NM, 2001

8. Shurin G.V., **Yurkovetsky Z.R.**, Shurin M.R. Suppressed maturation of dendritic cells in cancer is mediated by down-regulation of CD40 expression. Keystone Meeting "Dendritic cells'. Taos, NM, 2001.

9. **Yurkovetsky Z.R.**, Gambotto A., Kim S.H., Shurin M.R., Robbins P .D. Intratumoral administration of Ad-CD40L or DCY/CD40L elicited effective antitumor immunity in mice. 4th annual ASGT meeting, 2001.

Meetings Attended:

Intratumoral administration of adeno CD40L or dendritic cells overexpressing CD40L elicited effective antitumor immunity in mice fl. Era of Hope, DOD Breast Cancer Research Program Meeting, 2002.

Serkan Alkan (Dr. Christine Milcarek, advisor), Biochemistry and Molecular Genetics Program. Serkan works on the influence of hnRNP F and H on gene expression. The nuclear RNA binding proteins hnRNP F and H' have been shown to influence mRNA processing in many studies. The hnRNP F competes with polyadenylation factors and negatively influences expression. He is pursuing the mechanism of action of these proteins on cancer. By using RNA electromobility shift assays, he narrowed down the binding region of hnRNP F and H' on SV 40 late pre-mRNA to a 14 nucleotide Guanine rich region. Using serial RNA mutation analyses he concluded that the five consecutive Guanines in the sequence are necessary and sufficient for efficient hnRNP F and H' binding. He also verified that this binding is a result of specific RNA-protein interactions. Structure function analyses of hnRNP F are currently being conducted. He identified induced and repressed genes in murine plasmacytoma (AxJ) vs lymphoma cells (A20) by using microarray technology. He also did microarray analysis to compare the gene expression of hnRNP F, H' and empty vector transfected AxJ cells. The regulation of these will be pursued. He conclude that the hnRNP proteins F and H' can influence gene expression and that their differential expression in a variety of tissues and cell states may be ubiquitous regulators of mRNA processing.

Meetings Attended:

1. Poster presentation at the 3' Processing meeting, Cold Spring Harbor NY, August 21-25 2001.
2. Poster presentation at the RNA Meeting, Wisconsin Madison May 28- June 2 2002.
3. Poster presentation at the DOD meeting in Orlando, September 2002.
4. Poster presentation at the 4' Processing meeting, Cold Spring Harbor NY, 2003.

Rafael Flores (Dr. Penelope Morell, advisor), Immunology Graduate Program. Rafael was funded for one year and his funding was just renewed for another year. A project that he has pursued this past year was the examination of the ability of murine bone marrow derived DCs to adopt either a DC1 or a DC2 functional phenotype. He conducted a preliminary study utilizing an *in vitro* protocol. Bone marrow cells from BALB/c mice were grown in GM-CSF for four days and then matured for 18 hours in the presence of TNF-a, PGE_2, or IL-4. Our results show that BM cells grown in GM-CSF and matured in the presence of TNF-a + PGE_2 were high producers of IL-12p70 whereas IL-4 matured DCs were low producers of IL-12p70. In addition to the high IL-12p70 production, the TNF-a + PGE_2 matured DCs exhibited a significantly higher level of CD86 than DCs matured with any other cytokine combination of cytokines. This pattern was also observed in experiments conducted with the BM cells of C57BL/6 and the NOD. In each mouse strain tested, IL-4 matured DCs were low producers of IL-12p70. Presently, he is analyzing the T cell polarizing ability of the different DC subsets. This work has importance fo induction of immune responses against tumors.

Meetings Attended:

2002 FASEB-AAI Conference- Abstract Title "In Vitro Generation of Murine Bone Marrow Derived Dendritic Cell Types 1 and 2" Poster Presentation.

Abstracts:

Flores, RR, Hariri M, Morel, PA. Plasmacytoid DCs in the NOD mouse express reduced levels of LY-6c. 2003 FASEB J. 17:C39, Abstract #34.4.

Flores RR, M Feili-Hariri, P Kalinski, PA Morel. In vitro generation of murine bone marrow-derived dendritic cells type 1 and 2. 2002. FASEB J 16:A1231 Abstract # 929.14.

Nehad Alajez (Dr. Olivera J. Finn, advisor), Immunology Graduate Program. Nehad was funded for one year and his finding was extended for another year. He works on immunogenethrapy of breast cancer. MUC1 glycoprotein is overexpressed on the surface of a variety of epithelial tumors, most notably breast cancer, and has been under investigation as a target for immunotherapy. Cytotoxic T cell clones were generated from cancer patients that recognized MUC1 on the surface of tumor cells in an MHC-unrestricted manner. The T cell receptor (TCR) was cloned from one such clone (MA) and a two-chain (tc) and single-chain (sc) constructs were successfully expressed on the surface of a variety of cell lines. A secreted form of the scTCR receptor was expressed in 293 cells. Our Preliminary biacore data demonstrated the interaction between scTCR and tumor MUC1.The function of the scTCR was tested in vivo when a group of SCID mice were reconstituted with BM cells transduced with the scTCR amphotropic retroviral supernatant and challenged with MUC1-expressing human tumor cell lines. Tumor growth in mice reconstituted with TCR-transduced BM cells was significantly slower than that seen in the control group. The safety and efficacy of this approach are being tested in MUC1 transgenic mice. This strategy represents potentially efficacious gene therapy/immunotherapy for MUC1-expressing breast cancers. A non-MHC restricted TCR will make this treatment applicable to all cancer patients regardless of HLA type.

Publications:

1. Vlad A, Candelora-Kettel J, **Alajez NM**, Carlos CA, Finn OJ. MUC1 Immunobiology: from Discovery to Clinical Applications. Advances in Immunology. In press, 2003.

Meetings Attended:

1- Poster presentation at Experimental Biology meeting, New Orleans, Louisiana, April 20-24, 2002
Title: Cancer immunogene therapy using MUC1-specific MHC-unrestricted T cell receptor.

2- Platform and poster presentation at the Era of Hope meeting for the DOD Breast Cancer Research Program, Orlando, FL, September 25-28, 2002.
Title: Cancer immunogene therapy using MHC-unrestricted MUC1-specific T cell receptor.

Oral presentation at the American Association of Immunologist annual meeting, Denver, CO., May 2003
Title: MHC-unrestricted MUC1-specific T Cell Receptor for Cancer Immunotherapy

Alexander Ducruet(John Lazo, advisor), Pharmacology Graduate Program. Alexander works on targeted drug therapy for breast cancer. Cdc25A has oncogenic and anti-apoptotic activity and is overexpressed in numerous human tumors, including breast carcinomas. Cdc25A undergoes both positive and negative regulatory phosphorylations. For example, ionizing radiation or ultraviolet light exposure induce Cdc25A phosphorylation that targets it for degradation. Cdc25A can also be phosphorylated by Cdk2, Raf1, and Pim-1, resulting in increased phosphatase activity. Treatment of human tumor cells with the cdk inhibitor roscovitine (10 μM for 24 hr) resulted in a 3-fold increase in Cdc25A protein levels in HeLa cervical carcinoma cells and a 5-fold increase in Cdc25A protein levels in MCF-7 breast adenocarcinoma cells. Similarly, a 24 hr treatment of HeLa cells with 100 μM olomucine, a structurally related cdk inhibitor with reduced potency, increased Cdc25A levels 3-fold. Further examination revealed that Cdc25A protein levels were affected in a concentration- and time-dependent manner, increasing as early as 30-60 min following compound exposure. A genetic approach was employed to determine the cdk activity that was responsible for

this effect. Transfection of HeLa cells with a dominant-negative Cdk2 increased Cdc25A levels while no increase was seen with either a dominant-negative Cdk1 or Cdk3. These results support the hypothesis that Cdk2-mediated phosphorylation of Cdc25A decreases Cdc25A protein stability. Cdks are attractive therapeutic targets and cdk inhibitors are advancing into clinical trials. Nonetheless, the results suggest that elevated Cdc25A protein levels may be a potential negative side effect of Cdk2 inhibition, considering the oncogenic and anti-apoptotic potential of Cdc25A.

Publications:

1. Lazo, J. S., Aslan, D. C., Southwick, E. C., Kooley, K. A., **Ducruet, A. P.**, Joo, B., Vogt, A. and Wipf, P. Discovery and biological evaluation of a new family of potent inhibitors of the dual specificity protein phosphatase Cdc25. J. Med. Chem. 44: 4042-4049, 2001.

2. Lyon M. A., **Ducruet, A. P.**, Wipf, P. and Lazo, J. S. Dual specificity phosphatases as targets for antineoplastic agents. Nature Reviews Drug Discovery 1:961-976, 2002.

3. **Ducruet, A. P.** and Lazo, J. S. Regulation of Cdc25A half-life in interphase by Cyclin-dependent kinase 2 activity. J. Biol. Chem. 278: 31838-31842, 2003.

4. Lazo, J. S., **Ducruet, A. P.** and Koldamova, R. P. Sleuthful pharmacology. Mol. Pharm. 64: 199-201, 2003.

Abstracts:

Poster: Elevated Cdc25A Protein Levels in Response to Cyclin-Dependent Kinase Inhibition. Era of Hope Department of Defense Breast Cancer Research Program Meeting Proceedings Vol. III, Abstract P51-9, 2002.
Poster: Regulation of Cdc25A protein levels in human tumor cells by Cyclin-dependent kinase 2 activity. Proceedings American Association of Cancer Ressearch: (2nd ed.) 44: R1105, 2003.

Meetings Attended:

Gordon Research Conference on Chemotherapy of Experimental and Clinical Cancer, Colby-Sawyer College, New London, NH (July 14-19, 2002). Poster: Elevated Cdc25A Protein Levels in Response to Cyclin-Dependent Kinase Inhibition. Poster selected by attendees for informal oral presentation on final evening of conference)

Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orange County Convention Center, Orlando, FL (September 25-28, 2002). Abstract Submitted: Elevated Cdc25A Protein Levels in Response to Cyclin-Dependent Kinase Inhibition. Abstract selected for Poster Presentation)

Jennifer Johnson (Jean Latimer, advisor), MD., PH.D. student, Biochemistry and Molecular Genetics Program. Jennifer hypothesized that a decrease in the capacity of cells to perform Nucleotide Excision Repair (NER) has an etiological role in the formation of breast cancer. Preliminary data generated using the functional assay Unscheduled DNA Synthesis (UDS) showed that 100% of stage I sporadic breast tumors have a deficiency in global genomic repair relative to breast reduction. This work was completed on primary cultures generated in this lab using a unique tissue culture medium and technique. As a part of this project, she has also compared the repair capacity of these primary tumor cultures with 5 commercially available breast cancer cell lines and showed that their repair capacity is greatly increased relative to the primary cultures.

Abstract:

Kelly, C.M., **Johnson, J.M.,** Wenger, S.L., Vogel, V.,G., Kelley, J., Johnson, R., Amortequi, A., Mock, L., Grant, S.G. and Latimer, J.J. Analysis of functional DNA repair in primary cultures of the non-tumor adjacent breast identifies two classes of breast cancer patient. 94th Annual Meeting of the American Association for Cancer Research, Washington, D.C..(2003) Proceedings of the American Association for Cancer Research **44**: 974-975.

APPENDIX TO THE SUMMARY

1) Key research accomplishments:

- Promoters of the genes encoding two human arginase isozymes (types I and II) were cloned, their transcription start sites mapped, and the DNA regulatory elements and transcription factors involved in induction of arginase I by IL-4 and cAMP and of arginase II by bacterial lipopolysaccharide (LPS) and camp identified.

- A new anti-tumor therapy that was identified in year 3, based on DC/CD40L, was further tested and confirmed.

- The role in the control of cell differentiation of a new set of genes that was preliminarily identified in year 3, as alternatively expressed in cancer, was further evaluated and confirmed.

- Additional characterization of dendritic cell subsets was obtained that can influence immune responses towards type 1 (protective from tumor) or type 2 (not protective).

- A new reagent that was created for immunotherapy/gene therapy of breast cancer, a retroviral vector expressing a tumor-specific T cell receptor, was tested in vivo and found to protect from cancer challenge.

- Further evaluation was performed on Cdk2 inhibitors for targeted tumor therapy, with the results bringing a cautionary note about potential negative side effects.

2) Reportable outcomes

- Two trainees have completed their training and are scheduled to defend their Ph.D. thesis in December 2003.

- Four papers were published or are in press, authored or co-authored by the trainees.

- Three chapters were published

- Trainees authored 20 conference abstracts

3) Three copies of the published papers are included.

Running title: Immunobiology of MUC1

MUC1 IMMUNOBIOLOGY: FROM DISCOVERY TO CLINICAL APLICATIONS

Anda M. Vlad, Jessica C. Kettel, Nehad M. Alajez, Casey A. Carlos and Olivera J. Finn

Department of Immunology, University of Pittsburgh School of Medicine, E1040 Biomedical Science Tower, Pittsburgh PA 15261, USA

I. ABSTRACT

MUC1 glycoprotein is expressed on the luminal surface of most polarized epithelial cells and overexpressed over the entire cell surface of most adenocarcinomas. The cancer-associated MUC1 is structurally different from MUC1 on normal cells due to changes in glycosylation that result in the synthesis of tumor-specific glycoforms bearing novel T and B cell epitopes. Thus, MUC1 glycoprotein meets the criteria of a tumor specific antigen.

Vaccination with MUC1 is currently being explored for immunotherapeutic purposes and should ideally elicit IgG antibodies, strong helper and cytotoxic T cell responses and long-lasting antitumor immunity. Various vaccine formulations have been tested to date in preclinical animal models. Promising vaccines have also been tested in patients in phase I/II clinical trials. Results from these studies, successes and/or limitations of current therapeutic MUC1 vaccines as well as the potential of MUC1 vaccines for cancer prevention are reviewed.

II. INTRODUCTION

For more than a decade, tumor immunologists have focused their efforts on discovering tumor-associated antigens, as a first step towards the design of an effective cancer vaccine. To date, approximately 70 MHC class I and II-associated tumor antigens have been described, while more than 1,700 have been identified by antibodies in cancer patients (Yu and Restifo, 2002). However, it has become increasingly evident that this growing list of putative tumor-associated antigens will need to be supplemented with greater understanding of their molecular nature and mechanisms of action in order to validate them as suitable targets for tumor immunotherapy.

In this review we will highlight studies on MUC1, one of the first tumor antigens shown to be a target for human tumor-specific T cells and thus a valid target for immunotherapy. MUC1 is a member of the mucin family of molecules. It is expressed on the luminal surface of most polarized epithelial cells and overexpressed over the entire cell surface of most adenocarcinomas. Cancer-associated MUC1 is different from MUC1 on normal cells. During tumor progression there are changes in glycosylation that result in the synthesis of tumor-specific glycoforms bearing novel T and B cell epitopes. Thus, MUC1 glycoprotein meets the criteria of a tumor specific antigen and is currently employed in vaccines under investigation in several clinical trials.

Research on MUC1 has been reported in over 700 publications in the last five years, with the majority of these publications being focused on MUC1 immunobiology. These numbers, illustrating the interest in this molecule as an important tool in cancer research, also indicate the amplitude of the ongoing efforts to further explore the basic mechanisms behind its immunogenicity and its suitability as a target antigen for cancer treatment and prevention. We will briefly describe here the key research efforts that elucidate MUC1structure and biosynthesis pathways; however, our emphasis will be on the most recent studies that mark progress towards a better understanding of what makes MUC1 a tumor antigen, what kind of immune responses this molecule can trigger and how various immune effector mechanisms can be manipulated for therapeutic purposes.

III. HISTORY OF MUC1, THE PIONEER MEMBER OF THE MUCIN FAMILY

MUC1 was first identified in the milk fat globule membrane fraction and described as a protein rich in serine, threonine, proline, glycine, and alanine (Shimizu and Yamauchi, 1982). It was found to contain a high percentage of O-linked carbohydrates that accounted for about 50% of its molecular weight. In 1987, Gendler and colleagues were able to clone a fragment from this first human mucin gene by screening a mammary tumor cell line MCF-7 cDNA library using antibodies raised against a chemically deglycosylated form of milk mucin (Gendler *et al.*, 1987). The cloned gene, located on chromosome 1q21 (Dekker *et al.*, 2002), was sequenced and found to consist of numerous 60 base pair tandem repeats (Gendler *et al.*, 1987; Siddiqui *et al.*, 1988). Subsequently, cDNA encoding for splice variants of mucin were cloned from breast carcinoma cell lines (Ligtenberg *et al.*, 1990), from human breast tumor tissue (Gendler *et al.*, 1990; Wreschner *et al.*, 1990), and from pancreatic tumors (Lan *et al.*, 1990). This first human mucin gene was given the name MUC1 to replace preexisting names that included polymorphic epithelial mucin (PEM), polymorphic urinary mucin (PUM), epithelial membrane antigen (EMA), episialin, and MAM-6 DF3 antigen.

The other members of the mucin gene family were numbered in the order they were identified: MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6-9, MUC11-13, and MUC15-17. All mucins have certain structural features in common. They all consist of a peptide core with O-linked glycans attached to serine and threonine residues. The protein core consists of a variable number of repeated sequences (tandem repeats) distinct to each mucin. Mucins can exist in a secreted form (gel-forming), membrane-bound form or both. MUC1, MUC3-4 (Moniaux *et al.*, 2000; Williams *et al.*, 1999b), MUC12-13 (Williams *et al.*, 1999a; Williams *et al.*, 2001), and MUC15-17 (Gum *et al.*, 2002; Pallesen *et al.*, 2002; Yin *et al.*, 2002) can be expressed as membrane-bound glycoproteins. These membrane-bound mucins have a transmembrane domain that facilitates their anchoring in the membrane lipid bilayer. The rest of the mucins can only be expressed in soluble form.

MUC1 is normally present on the apical surface of most polarized epithelial tissues of the respiratory, genitourinary tract and digestive system. It is also expressed on normal breast ducts. MUC1 is overexpressed on the majority of adenocarcinomas of the breast, lung, colon, pancreas, stomach, prostate, and ovary (Ho *et al.*, 1993). MUC1 expressing cancers account for about 70% of new cancer cases expected in the year 2003 (Jemal *et al.*, 2003). The forms of MUC1 produced by tumor cells differ in many ways from normal MUC1. As an epithelial cell undergoes malignant transformation, it loses the normal apical-basolateral polarity and begins to express MUC1 on the entire cell surface. The level of expression also increases and a soluble form of MUC1 can be found in the serum of cancer patients.

Similar to MUC1, other members of the mucin family also have an altered expression on different tumors. MUC4 is overexpressed in adenocarcinomas of the lung and expressed *de novo* in pancreatic and gastric cancers (Balague *et al.*, 1994; Buisine *et al.*, 2000; Nguyen *et al.*, 1996). In one report, about 29% of lung cancer patients had high titers of anti-MUC4 IgG and IgM antibodies (Hanaoka *et al.*, 2001). Overexpression of MUC6 and *de novo* expression of MUC2, MUC4, and MUC5AC has been demonstrated on the surface of adenocarcinomas of the pancreas and on pancreatic tumor cell lines (Balague *et al.*, 1994). However, there is only limited information about the immunogenicity of these other mucins and prognostic significance of their altered expression is not known.

IV. STRUCTURE, BIOSYNTHESIS AND PHYSIOLOGY OF MUC1 IN HEALTH AND DISEASE

A. MUC1 structure and biosynthesis

Unlike the majority of mucins that are secreted from cells, MUC1 is expressed as both transmembrane and secreted form. Though it is encoded as a single protein, it is expressed as a type I transmembrane heterodimer. The two proteins that make up MUC1 differ greatly in size with most of the larger MUC1 fragment being composed of a tandemly repeated 20 amino acid sequence PDTRPAPGSTAPPAHGVTSA. This serine, threonine and proline rich sequence can be repeated up to 125 times in a single MUC1 molecule, commonly occurring between 41-85 times (Carvalho *et al.*, 1997; Gendler *et al.*, 1990). This region of the molecule is referred to as VNTR for variable number of tandem repeats.

The biosynthesis of MUC1 proceeds via distinct steps (Hilkens and Buijs, 1988). The newly synthesized protein receives several N-glycans adjacent to its transmembrane region following co-translational transfer of high-mannose glycans during synthesis in the endoplasmic reticulum. Within 1–2 minutes, while still in the endoplasmic reticulum, MUC1 undergoes proteolytic cleavage. Ligtenberg et al showed in 1992 that the 2 cleavage products remain non-covalently associated so that the smaller transmembrane fragment anchors the larger piece. A proteolytic cleavage site, FRPG/SVW, located 65 amino acids upstream of the transmembrane domain, was identified recently (Parry *et al.*, 2001). After cleavage, the precursors move through the Golgi where the N-glycans become more complex and O-glycosylation is started on the VNTR region. O-glycosylation increases the molecular weight dramatically within the first 30 minutes of synthesis. MUC1 becomes partially sialylated on its O-linked oligosaccharides before leaving the Golgi as a premature form. Completely and incompletely sialylated MUC1 are both expressed on the cell surface (Litvinov and Hilkens, 1993). Trafficking of MUC1 to the cell surface is thought to be controlled by at least two signals, one contained in Cys-Gln-Cys motif at the junction of the MUC1 tail and transmembrane domains, and a second in the extracellular domain but outside of the VNTR region (Pemberton *et al.*, 1996).

To become fully sialylated, the premature form recycles several times from the cell surface to the trans-Golgi and back to the surface. Complete sialylation occurs within 3 hours (Hilkens and Buijs, 1988). The recycling of MUC1 is constitutive so that even after full sialylation, a mature MUC1 molecule completes 10 cycles before being released from the cell, approximately 24 hours after synthesis. MUC1 on the surface of normal cells is completely sialylated while on tumor cells the surface MUC1 is a combination of completely and incompletely sialylated molecules. It was suggested that this is due to greater abundance of MUC1 on tumor cells and/or less efficient sialylation process compared to normal cells (Litvinov and Hilkens, 1993).

NMR studies using peptides composed of one to three tandem repeats have shown that, as the number of repeats increases, the structure of MUC1 becomes more ordered. Indeed, intrinsic viscosity measurements indicate that the peptide composed of three repeats has a rod-like structure (Fontenot *et al.*, 1993), suggesting that MUC1 on the cell surface would project outwards rather than exist in a globular shape. Further NMR studies established that in each repeat, the APDTR sequence, to which antibodies have been raised, exists on a protruding knob-like structure on the MUC1 backbone (Fontenot *et al.*, 1995b). When multiple repeats are examined, the overall effect is a rod with evenly spaced knobs throughout the entire VNTR region. Most antibodies against MUC1 bind to this epitope making it immunodominant on the native MUC1 molecule (Price *et al.*, 1998).

Because of the large number of repeats in the VNTR region, MUC1 can extend 300-500 nm above the cell surface, towering over other cell surface molecules. Twenty-five percent of the amino acids in the VNTR region are either serine or threonine that can be O-glycosylated. On either side of the VNTR region are several degenerate repeats (Engelmann *et al.*, 2001; Ligtenberg *et al.*, 1990). Recently, Engelmann, et al. provided genetic evidence of variation in the 20 amino acid sequence within the VNTR domain. By sequencing PCR products followed by minisatellite variant repeat analysis of the 5' and 3' peripheral areas of the VNTR region in 33 samples taken from normal and cancerous cells, they found that the same sequence variation consistently occurred in the same repeats. This indicates that the variation predates the duplication event that has led to the elongated VNTR domain. The proline (cca) in position 13 of the tandem repeat sequence PDTRPAPGSTAPPAHGVTSA could be altered to glutamine (caa), alanine (gca) or threonine (aca), possibly generating an additional glycosylation site. The other location of sequence change is in the immunodominant epitope, APDTR, in which the DT (gacacc) is substituted with ES (gagagc). This was the most commonly seen sequence variation within the diverse population studied. However, in the majority of samples this variation was found in only four of the 24 repeats sequenced from each of 33 samples. This particular variation within the immunodominant peptide sequence could be regarded as a source of additional epitopes with immunogenic potential; nevertheless, since this mutated (ES) sequence is less commonly seen than the conserved DT sequence found in the majority of repeats, one should expect the majority of responses to be directed towards the highly conserved and overwhelmingly abundant tandem repeat sequences.

The smaller piece (~ 20 kDa) of MUC1 contains a short extracellular portion, a transmembrane region and a short intracellular tail. In its extracellular domain are sites for N-linked glycosylation (Gendler *et al.*, 1990; Wreschner *et al.*, 1990). The transmembrane region carries cysteines that may be used for fatty acid acetylation to help anchor MUC1 in a cell's membrane (Ligtenberg *et al.*, 1990). In the cytosolic tail are potential sites of phosphorylation and intracellular protein binding that prompted research into the possibility that MUC1 could be a signaling molecule. This was especially of interest because the exact function of MUC1 is still not known. Alternative splicing of MUC1 mRNA can lead to multiple forms being expressed by a single cell type. When the full-length cDNA and genomic organization of MUC1 were initially published they showed that two different amino terminal signal sequences could be produced. The longer form, referred to as MUC1/A has an additional 27 base pairs when compared to MUC1/B (Ligtenberg *et al.*, 1990; Wreschner *et al.*, 1990). Whether MUC1/A or MUC1/B is produced depends on whether a guanine or adenine is present 8 nucleotides downstream of exon 1, in the first intron. When guanine is present, the longer MUC1/A is synthesized and the number of repeats is higher. Conversely, when adenine is present there are fewer repeats and the shorter isoform MUC1/B is made (Ligtenberg *et al.*, 1991).

Soluble MUC1 is found in human milk (Patton, 2001; Peterson *et al.*, 2001) and in barely detectable amounts in the serum of healthy men and women (Croce *et al.*, 2001b; McGuckin *et al.*, 1994). This form may be produced when a splice donor site downstream of the VNTR region is not used during transcription, allowing translation of a stop codon prior to the transmembrane region (Wreschner *et al.*, 1990). In 1996, a monoclonal antibody was generated against this spliced out peptide sequence (Smorodinsky *et al.*, 1996). With this antibody, soluble MUC1 was detected in supernatants of cancer cell lines and in sera of cancer patients. However, mouse mammary epithelial cells transfected with full-length human MUC1 in which alternative splicing could not occur (Boshell *et al.*, 1992), still produced soluble MUC1 lacking the cytosolic tail.

This supports a second mechanism for production of soluble MUC1 that proposes that MUC1 is released from the surface of cells by proteolytic cleavage (Hilkens, 1991). TACE (TNFα converting enzyme) is considered the likely protease responsible for the cleavage (Thathiah *et al.*, 2003). Other potential mechanisms are cleavage by external proteases or simple dissociation of the heterodimeric complex. The involvement of external proteases is not likely, given that addition of proteolytic inhibitors has no effect on the amount of soluble MUC1 (Julian and Carson, 2002). Simple dissociation seems unlikely as well, given that MUC1 remains a stable heterodimer during repeated recycling through the cell for further glycosylation and sialylation (Ligtenberg *et al.*, 1991; Litvinov and Hilkens, 1993). Furthermore, when a mutated form of MUC1 that lacks the site of initial cleavage is expressed as a single protein, it is still released from the cell (Ligtenberg *et al.*, 1992).

1. O-linked glycosylation of MUC1 in normal epithelia

Because of the differences in MUC1 glycoforms expressed on normal and cancerous epithelium, there has been a great effort to understand MUC1 O-linked glycosylation. The majority of MUC1 glycosylation occurs in the VNTR region on the two serines and/or three threonines in each repeat. The most common carbohydrate additions to these amino acids is a core 2 structure, an N-acetyl galactose that has a galactose branching from its third carbon and N-acetyl glucose branching from its sixth carbon. In normal MUC1, these branches are elongated and effectively cloak the peptide backbone. Only a minor fraction of normal MUC1 glycosylation consists of core 1 additions (Hanisch and Muller, 2000). The core 1 structure is an N-acetyl galactose that has only the galactose branching from its third carbon, no addition to carbon 6. This yields a less effective cloaking of the peptide backbone and is predominantly seen on the tumor form of MUC1.

While N-linked glycosylation occurs at known consensus sites, O-linked glycosylation motifs have not been identified. However, human GalNAc transferases responsible for initiating O-linked glycosylation on MUC1 have been studied *in vitro* (Wandall *et al.*, 1997) and their *in vivo* products analyzed (Muller *et al.*, 1997) using recombinant enzymes and MUC1 peptides. Regardless of whether the peptide contained one or five repeats PDTRPAPGSTAPPAHGVTSA, only three of the five Ser/Thr sites per repeat were glycosylated. No glycosylation was seen on the Ser in GVTSA or Thr in DTR. Interestingly, the enzyme kinetics varied for the site being glycosylated, *e.g.* GalNAc-T2 being the fastest to glycosylate ST in GSTAP but slowest on the T in GVTSA (Wandall *et al.*, 1997). In human milk, however, all five potential sites could be glycosylated with an average of 2.7 sites per repeat (Muller *et al.*, 1997). The discrepancy between *in vitro* and *in vivo* work could be attributed to additional GalNAc transferases working *in vivo* and an enhancing effect of previous glycosylation on subsequent glycosylation. This was demonstrated with a recombinant GalNAc-T4 transferase that could glycosylate Ser in GVTSA and Thr in PDTR but only if the peptide had been previously glycosylated (Bennett *et al.*, 1998). Furthermore, studies with transferases GalNac-T1, -T2 and -T3 showed that *in vitro* glycosylation occurred differently on single MUC1 tandem repeat peptides depending on how many sites were previously glycosylated.. Distant and neighboring effects on subsequent glycosylation as well as enzymatic competition between core synthesizing enzymes and transferases could explain the MUC1 glycosylation differences between normal and cancer cells (Hanisch *et al.*, 1999). Further studies are continuing to explore this highly dynamic regulation of O-glycosylation (Dalziel *et al.*, 2001; Hanisch *et al.*, 2001).

2. O-linked glycosylation of MUC1 in tumor cells

The most striking difference between normal MUC1 and tumor MUC1 is in their glycosylation (Burchell *et al.*, 2001; Hanisch and Muller, 2000). Changes in the relative and total levels of glycosyltransferases in tumor cells are largely responsible for this aberration (Beum *et al.*, 1999). Prematurely terminated carbohydrates found on tumor MUC1 include the Thomsen-Friedenreich antigen (Galβ1-3GalNAc-Thr/Ser), Tn antigen (GalNAc-Thr/Ser), and sialyl-Tn antigen (Sialylα2-6GalNAc-Thr/Ser). Many of the antibodies generated by cancer patients have been found to be specific these short carbohydrates linked to the MUC1 backbone. Shorter carbohydrate chains also allow the peptide backbone of the VNTR region, and especially the immunodominant knobs to be exposed and recognized by MHC-unrestricted T cells and antibodies (Barnd *et al.*, 1989; Fontenot *et al.*, 1995b).

In 2001, Obermair, looking at cervical carcinoma cells, found two novel MUC1 splice variants (Obermair *et al.*, 2001). These were shorter than the variants described for normal MUC1 and were named MUC1/C and MUC1/D. Both are the result of alternative splice acceptor sites when joining exons one and two. Splice variants (MUC1/Y, MUC1/X, and MUC1/Z) of MUC1 lacking the VNTR region have also been reported. MUC1/Y transcripts and protein were found in primary breast cancer tissue (Zrihan-Licht *et al.*, 1994). MUC1/X (Baruch *et al.*, 1997) and MUC1/Z (Oosterkamp *et al.*, 1997), both larger than MUC1/Y by 18 amino acids, were reported in cancer cell lines. Polymorphisms in the length of the VNTR region have also been studied in patients with gastric carcinoma and two of the premalignant states associated with this disease. The allele that encodes the short VNTR region was found to be highly associated with both premalignant conditions and with susceptibility to gastric carcinoma. Homozygosity for the long allele was associated with one of the premalignant states but not with carcinoma. The heterozygous state was found to offer the most protection from disease (Carvalho *et al.*, 1997; Silva *et al.*, 2001).

B. MUC1 Physiology

The physiologic role of normal MUC1 is still undetermined. As a member of the mucin family, its assumed role, both in its secreted and membranous forms, is the lubrication of epithelial and ocular surfaces (Gipson and Inatomi, 1998). MUC1 can bind to pathogens at the epithelial surface through its O-linked carbohydrates (DeSouza *et al.*, 1999; Lillehoj *et al.*, 2001; Schroten *et al.*, 1992; Yolken *et al.*, 1992). This interaction has been hypothesized to either prevent the pathogen access to the cell membrane or conversely, to aid in the adherence and subsequent infection of the host tissue. It acts as a barrier to embryo implantation in multiple species and may play a part in the maintenance of pregnancy (Bowen *et al.*, 1996; Croy *et al.*, 1997; DeSouza *et al.*, 1998; Hewetson and Chilton, 1997; Hild-Petito *et al.*, 1996; Hoffman *et al.*, 1998; Meseguer *et al.*, 1998; Surveyor *et al.*, 1995). During embryogenesis, MUC1 is induced in developing epithelial tissue but not in squamous tissue (Braga *et al.*, 1992; Guzman *et al.*, 1996; Shin *et al.*, 2000). The yeast homologue of MUC1 is necessary for pseudohyphal differentiation and invasive growth in yeast (Lambrechts *et al.*, 1996). Human MUC1 induces alterations in cellular morphology of transfected mammalian cells (Hudson *et al.*, 2001). However, whether this expression is simply correlated with epithelial development in mammalian tissue or whether it plays a role in the spatial development of glandular tissue is still under investigation. More recently, it has been suggested that MUC1 may affect erythropoiesis since it is temporally expressed in erythroblasts (Rughetti *et al.*, 2003). This list is by no means comprehensive and new roles for MUC1 are still being defined.

One of the best ways to gain a global understanding of the importance of a molecule is through the study of knockout animal models. A muc1 (the mouse MUC1 homologue) knockout mouse was created on the C57BL/6 background (Spicer *et al.*, 1995). Interestingly, in a transgenic germ free environment, these mice developed normally into fertile and healthy adults. However, primary breast tumors induced by polyoma middle T antigen grew significantly slower in these muc1 knockout mice (Danjo *et al.*, 2000; Spicer *et al.*, 1995). The same investigators had previously shown that mucl plays a role in the formation of intestinal mucus in a cystic fibrosis mouse/muc1 double knockout model (Parmley and Gendler, 1998). The muc1 knockout mouse has been reported to have increased susceptibility to bacterial conjunctivitis, vulvovaginitis, and decreased litter size but only when housed in a specific pathogen free vivarium with exposure to endogenous mouse flora (Croy *et al.*, 1997; DeSouza *et al.*, 1999; Kardon *et al.*, 1999). From this work it is clear that although muc1 has some unique functions, there must also be other proteins that can compensate for its role in development.

Recent studies have shown that MUC1 is expressed on the surface of T cells after activation. MUC1 expression by T cells has been documented by immunohistochemistry (Delsol *et al.*, 1984), flow cytometry (Agrawal *et al.*, 1998a; Chadburn *et al.*, 1992; Chang *et al.*, 2000; Correa *et al.*, 2003; Fattorossi *et al.*, 2002; Wykes *et al.*, 2002), RT-PCR (Agrawal *et al.*, 1998a; Chang *et al.*, 2000; Correa *et al.*, 2003; Fattorossi *et al.*, 2002), Northern blotting (Chang *et al.*, 2000) and confocal microscopy (Correa *et al.*, 2003). Most studies have shown MUC1 expression on activated and not resting T cells (Agrawal *et al.*, 1998a; Chadburn *et al.*, 1992; Chang *et al.*, 2000; Correa *et al.*, 2003; Fattorossi *et al.*, 2002; Wykes *et al.*, 2002). The function of MUC1 on activated T cells has not been clearly defined but it has been suggested that it may play a role in immune regulation (Agrawal *et al.*, 1998a) and modulation of cell-cell interaction (Correa *et al.*, 2003). Indications of its role *in vivo* may come from determining where MUC1 expressing T cells are found in the body. Correa et al. (Correa *et al.*, 2003) detected MUC1 on 10% of T cells in the synovial fluid of patients with rheumatoid arthritis. No MUC1+ T cells could be detected in the patient's blood. This suggests the possibility that MUC1 on activated T cells is used during migration into the inflamed joint. This finding opens up a novel application for anti-MUC1 vaccination. Antibodies against MUC1 that would be generated through vaccination could be expected to hinder T cell entry into the arthritic joint. Since activated memory T cells are the dominant cell type present in synovial tissue (Kohem *et al.*, 1996) and memory T cells express MUC1 (Correa *et al.*, 2003), MUC1 vaccination could reduce inflammation in a T cell specific manner.

A frequent question regarding the expression of MUC1 on activated T cells is whether an immune response elicited by MUC1 cancer vaccines would target activated T cells. This is highly unlikely to happen when immune responses elicited by vaccines are focused on tumor-specific forms of MUC1. These immunogens generate immune cells specific for epitopes present only on tumor cells. Activated T cell express the glucosyltranferase enzymes that lead to long, highly branched polysaccharides on MUC1 (Correa *et al.*, 2003; Piller *et al.*, 1988) and do not present the same MUC1 epitopes found on tumor cells.

Most of the studies on the function of MUC1 involve the extracellular domain of the molecule; however, its cytosolic tail is also important. The cytosolic tail of MUC1 is well conserved among many species (Pemberton *et al.*, 1996). Seven tyrosines are present in that region (Wreschner *et al.*, 1990) and available for phosphorylation. According to work done with tumor cells, MUC1 transfected cells, or CD8/MUC1 chimeric fusion protein expressing cells, these tyrosines can be phosphorylated (Meerzaman *et al.*, 2000; Pandey *et al.*, 1995; Quin and

McGuckin, 2000; Zrihan-Licht *et al.*, 1994). In a variety of cells and conditions MUC1 co-immunoprecipitates several intracellular protein(s) (Pandey *et al.*, 1995; Quin and McGuckin, 2000; Schroeder *et al.*, 2001; Yamamoto *et al.*, 1997; Zrihan-Licht *et al.*, 1994). Associations between MUC1 and the c-Src tyrosine kinase have been reported (Gonzaez-Guerrico *et al.*, 2002; Li *et al.*, 2001a; Li *et al.*, 2001b) as well as activation of ERK1/2 *in vivo* (Schroeder *et al.*, 2001) and indirect activation of ERK2 via Ras and MEK *in vitro* (Meerzaman *et al.*, 2000). MUC1 also interacts with the catenin, p120, increasing the nuclear localization of p120 (Li and Kufe, 2001). Association of MUC1 with transmembrane tyrosine kinases, epidermal growth factor receptor, erbB2, erbB3 and erbB4 has been shown in vivo (Schroeder *et al.*, 2001).

MUC1 in tumor cells has been associated with β-catenin (Schroeder *et al.*, 2001; Yamamoto *et al.*, 1997), a protein involved in cadherin-mediated cell adhesion. Further studies have shown that binding to β-catenin is affected by phosphorylation of the MUC1 tail. There is increased binding following MUC1 phosphorylation by protein kinase C δ (Ren *et al.*, 2002) but decreased binding to β-catenin following the action of glycogen synthase kinase 3β (Li *et al.*, 1998). Though the interaction between MUC1 and β-catenin has been proposed to explain the inhibitory effect of MUC1 expression on cadherin mediated adhesion (Carraway *et al.*, 2003), this is highly unlikely since tail-less mutants of MUC1 equally hinder binding (Wesseling *et al.*, 1995). Rather, inhibition is more likely due to the high degree of steric hindrance that MUC1 provides on the cell surface (Ligtenberg *et al.*, 1992), illustrated by experiments using MUC1 with varying numbers of repeats (Wesseling *et al.*, 1996; Wesseling *et al.*, 1995).

In hope of better understanding the role of MUC1 in metastasis of tumor cells, the study of tumor MUC1 in cell adhesion continues to be an active and important area of research. Tumor cell adhesion has been associated with the extent of phosphorylation of the MUC1 tail (Quin and McGuckin, 2000). As cells begin to adhere, MUC1 phosphorylation decreases over time indicating that motility and adherence are associated with MUC1 phosphorylation. How this occurs is debatable. Pandey et al., (Pandey *et al.*, 1995) showed that phosphorylated MUC1 associates with Grb2, an adapter protein involved in signaling pathways. However, this association could not be replicated by Quin et al., (Quin and McGuckin, 2000). The latter group did however co-immunoprecipitate with MUC1 a 60 kDa phosphorylated molecule as yet unidentified. Further work is needed to elucidate these interesting associations between MUC phosphorylation, interactions with other proteins inside the cell as well as the effect on adhesion.

1. Expression of MUC1 by tumor cells and its role in carcinogensis

MUC1 has been identified as a marker of preneoplastic conditions and of several chronic inflammatory diseases. Changes that occur during chronic inflammation include increases in the serum level of MUC1, the generation of antibodies to MUC1, and increases in the cell surface level of MUC1 on affected cells (Kohno, 1999; Nakajima *et al.*, 1998; Takaishi *et al.*, 2000). The antibodies to MUC1 found in patients with ulcerative colitis are found in chronic but not acute ulcerative colitis. Interestingly, these antibodies are specific for the peptide backbone of MUC1 and suggest that changes in MUC1 glycosylation may be occurring early in chronic inflammatory conditions as well as in cancer (Hinoda *et al.*, 1993). The surface expression of MUC1 is reported to be upregulated in preneoplastic conditions of virtually every tissue that gives rise to a MUC1 positive neoplasm (Adsay *et al.*, 2002; Arul *et al.*, 2000; Boman *et al.*, 2001; Buisine *et al.*, 2001; Cao *et al.*, 1999; Copin *et al.*, 2000; Jarrard *et al.*, 1998; Lopez-Ferrer

et al., 2001; Luttges *et al.*, 2002; Masaki *et al.*, 1999; Reis *et al.*, 1999). Whether the expression of MUC1 is part of the pathogenesis of cancer or inflammatory disease or whether it is simply a marker of the disease state is still under investigation.

MUC1 is expressed on virtually all adenocarcinomas as well as several other malignancies and numerous groups have been able to use MUC1 or the immune response to MUC1 as a marker of disease state (Brossart *et al.*, 2001). Its expression has been linked to a worse prognosis for many of these tumors (Ajioka *et al.*, 1996; Baldus *et al.*, 2002a; Baldus *et al.*, 2002b; Kraus *et al.*, 2002; Leroy *et al.*, 2002a; Leroy *et al.*, 2002b; Pinto-de-Sousa *et al.*, 2002; Sagara *et al.*, 1999; Sivridis *et al.*, 2002; Yamato *et al.*, 1999). There is also evidence that MUC1 enhances the metastatic abilities of tumor cells (Aoki *et al.*, 1998; Guddo *et al.*, 1998; Hiraga *et al.*, 1998; Tanimoto *et al.*, 1999; Utsunomiya *et al.*, 1998). Cancer patients often have an immune response to MUC1 manifested by low affinity cytotoxic T cells and low titer antibodies to MUC1. An immune response to MUC1, manifested through antibodies to MUC1 or MUC1 specific T cells, has been linked to a better overall prognosis.

Progression from chronic inflammation to preneoplastic and then neoplastic disease is sometimes a lengthy process. During this process, MUC1 undergoes changes that can render the molecule capable of triggering immune effector mechanisms. Identifying these changes and understanding how the immune effector mechanisms could be manipulated at preneoplastic stages through vaccination may constitute a first and important step towards the design of a vaccine for tumor prevention.

2. MUC1 expression and function on tumor cells

Since MUC1 is upregulated in preneoplastic and neoplastic conditions, it has been postulated that it may play a role in the growth and/or dissemination of tumor cells. One of the ways that MUC1 contributes to tumor growth is through its ability to affect cell-cell and cell-matrix adhesion (Ciborowski and Finn, 2002; Ligtenberg *et al.*, 1990; Wesseling *et al.*, 1996; Wesseling *et al.*, 1995). MUC1 can also hinder the function of the shorter adhesion molecules on the tumor cell surface through its large and rigid structure and thus serve as an anti-adhesive molecule. This inhibition of adhesion may be critical to the metastasis of cells from the primary tumor site. It could also explain the correlation between MUC1 expression and increased metastatic potential seen in some cancers. Conversely, MUC1 is able to bind to adhesion molecules, through its carbohydrate residues and its backbone, which may be important for tumor migration (McDermott *et al.*, 2001; Regimbald *et al.*, 1996; Tomlinson *et al.*, 2000). MUC1 also affects the efficiency of various anti-tumor immune response by preventing NK cell binding to tumor cells, suppressing T cell function, and affecting the ability of dendritic cells to function as antigen presenting cells (Agrawal *et al.*, 1998b; Fung and Longenecker, 1991; van de Wiel-van Kemenade *et al.*, 1993; Zhang *et al.*, 1997) . Our group has shown that a circulating form of MUC1 purified from cancer patients can bind to dendritic cells through the mannose receptor and most likely through other lectin receptors (Hiltbold *et al.*, 2000). We are currently investigating the functional consequences of this interaction with the dendritic cell.

V. MUC1 IMMUNOBIOLOGY

A. Naturally occurring immune responses to MUC1

1. In healthy humans:

The presence of anti-MUC1 antibodies of IgM and IgG isotypes as well as of circulating MUC1 antigen in sera from normal healthy women is well documented (Richards *et al.*, 1998). Agrawal et al (Agrawal *et al.*, 1995) have shown that MUC1-specific T cells can be primed during pregnancy, as T cells from biparous but not nulliparous women proliferated specifically in response to core MUC1 peptides. These findings could be explained by the fact that anatomical and physiological changes of MUC1-expressing organs (like the uterus and breast) during normal processes (like pregnancy and lactation) can prompt subtle changes in MUC1 production and can eventually trigger priming to MUC1 of immune effectors, like B cells and possibly T cells. Two recent studies (Croce *et al.*, 2001a; Croce *et al.*, 2001b) provide a good analysis of antibody responses in healthy women, correlated with their current or previous pregnancy/lactation status. Plasma measurements of free circulating MUC1 as well as of MUC1 complexed with antibodies in immune complexes showed elevated levels in pregnant women, compared to non-pregnant women. During pregnancy, there is a dramatic increase in MUC1 during the second trimester up to puerperium; by contrast, although the levels of immune complexes are gradually increasing, there is a drop in the levels of free anti-MUC1 IgG and IgM antibodies, which reach their lowest value at puerperium and then gradually increase after delivery. Lactation can also influence anti-MUC1 antibody production, since the titer of IgG isotype was significantly higher in the lactating group when compared to non-lactating women.

Despite the fact that these studies provide a good description of the spectrum of anti-MUC1 immune responses arising spontaneously to a self molecule, they do not identify any of the reacting epitopes. Further analyses of the (expectedly polyclonal) MUC1 epitopes are still needed. Moreover, the significance of this natural immunization with MUC1 remains to be elucidated. Epidemiological studies performed to date suggest a correlation between pregnancy and lower risk of developing breast cancer (Kalache *et al.*, 1993; MacMahon *et al.*, 1982). In that regard, we have reported a case of a long-term breast cancer survivor whose pregnancy might have triggered MUC1-specific immune response that prevented recurrence of tumor (Jerome *et al.*, 1997). The patient was diagnosed with breast tumor that was successfully removed. Five years later, she became pregnant and developed acute inflammatory cellulites in her breast. Breast tissue from this patient expressed the same MUC1 immunodominant epitope as presented by the original tumor. The subject had high titer of circulating anti-MUC1 IgM and IgG antibodies and a high frequency of MUC1-specific cytotoxic T lymphocytes (CTL) in the blood. She remained tumor-free for an additional 5 years of follow-up. It is possible that secondary immune responses against MUC1 were precipitated by pregnancy and prevented the recurrence of breast cancer. Importance of such natural immunity to MUC1 on the incidence of other cancers (like uterine and ovarian carcinomas) also remains to be addressed.

2. In cancer patients:

In addition to the above findings that suggest immunization to a self antigen under physiologic conditions, we and others have shown that anti MUC1 responses could also be triggered in cancer patients during growth of MUC1 positive tumors. In general, tumor cells are poorly recognized by the immune system of tolerance to self-antigens. Moreover, tumor cells can utilize a variety of mechanisms to evade recognition and to suppress cells of the immune system: downregulation of MHC class I (Zheng *et al.*, 1999), lack of costimulation (Banat *et al.*, 2001), loss of antigenic variants (Riker *et al.*, 1999), expression of FasL (Strand *et al.*, 1996), secretion of inhibitory cytokines (Beck *et al.*, 2001; Shurin *et al.*, 2002; Yang *et al.*, 2003) etc.

Despite these inhibitory mechanisms, given its characteristics that differentiate it from self, MUC1 made by tumor cells can still trigger, in cancer patients, humoral and cellular responses, although of low efficiency. Kotera and colleagues have reported anti-MUC1 IgM antibodies in more than 10% of sera from breast, colon, and pancreatic cancer patients (Kotera *et al.*, 1994). The presence of only IgM isotype in these sera indicated a T helper independent anti-MUC1 immune response. Petrarca and colleagues were able to isolate *in vivo*-primed B cells from the draining lymph nodes of 6 out of 12 patients with epithelial tumors (Petrarca *et al.*, 1999). These B cells secreted anti-MUC1 IgM and IgG antibodies when cultured *in vitro*. There was strong association between the ability to isolate B cells from these patients and the presence of anti-MUC1 IgM antibodies in their sera. A number of other reports demonstrated the presence of anti-MUC1 IgM and IgG antibodies in sera from patients with ulcerative colitis (Hinoda *et al.*, 1993), ovarian cancer (Snijdewint *et al.*, 1999), and colorectal cancer (Nakamura *et al.*, 1998). A strong correlation between the presence of anti-MUC1 antibodies in sera from cancer patients and better prognosis and patient survival has been reported in patients with pancreatic (Hamanaka *et al.*, 2003) and breast tumors (von Mensdorff-Pouilly *et al.*, 1996). Antibodies developed in cancer patients could bind to tumor antigens on the tumor cell surface and mediate complement dependent cytotoxicity and /or antibody-dependent cell mediated cytotoxicity. Such mechanisms are able to eliminate circulating tumor cells and micrometastases, as shown in preclinical and clinical studies by Zhang et al., (Zhang *et al.*, 1998).

MUC1 is also recognized by T cells. Cytotoxic T lymphocytes (CTLs) that recognized MUC1 on the surface of epithelial tumors were found in the draining lymph nodes of pancreatic cancer patients (Barnd *et al.*, 1989). It was then demonstrated that these CTLs recognize MUC1 on tumor cells in an MHC-unrestricted manner. These T cells have an α/β T cell receptor (TCR) and have $CD3^{+}CD8^{+}CD4^{-}$ phenotype. This MHC-unrestricted recognition of MUC1 could be blocked using an antibody against the immunodominant APDTRP epitope in the tandem repeat of the extracellular domain of tumor MUC1. CTLs that recognize MUC1 in an MHC-unrestricted manner were also established from draining lymph nodes of breast cancer patients (Jerome *et al.*, 1991) and from peripheral blood mononuclear cells (PBMCs) from patients with multiple myeloma (Takahashi *et al.*, 1994). Extensive studies of these T cells showed that they undergo similar intracellular signaling events as T cells that recognize conventional MHC/peptide complex (Magarian-Blander *et al.*, 1998). In fact, these T cells showed a large calcium influx when stimulated with beads coated with a MUC1 synthetic 100mer peptide carrying five repeats from the VNTR region and thus carrying five APDTR epitopes. This phenomenon of MHC-unrestricted recognition of MUC1 can be explained by the fact that MUC1 has multiple repeated epitopes that can cross link the TCR on T cells. This hypothesis was further supported when the NMR structure of unglycosylated synthetic MUC1 peptide was determined by Fontenot and colleagues (Fontenot *et al.*, 1995a). Their data revealed the presence of a knob-like structure protruding away from the backbone of each MUC1 tandem repeat with the sequence APDTR at the tip of this knob (described earlier in this article). MUC1-specific antibodies and T cells have increased accessibility to the immunogenic peptide backbone which exhibits shorter carbohydrate side chains on tumor cells, and are otherwise masked by heavy glycosylation in normal epithelial tissues (Hinoda *et al.*, 1998; Noto *et al.*, 1997).

CTL that recognize MUC1 peptides presented by MHC class I molecules have also been detected in patients. Antigenic peptides bound to MHC class I molecules are between eight-ten residues in length and are enclosed in a binding groove formed between α1 and α2 helices and the β-sheet platform of the MHC class I heavy chain. MHC alleles have preferences for

particular aminoacids (called anchors) at certain positions in the peptide (Rock and Goldberg, 1999). These anchor residues, most often described for high-affinity binding peptides, are necessary for stabilization of the peptide-MHC complex. Many peptides lacking the canonical anchor residues can also bind to MHC class I molecules, albeit with lower affinity, and be recognized by $CD8^+$ T cells. Most MHC-class I restricted MUC1 peptides are of that type. Eight-ten aminoacid peptides from the VNTR region were reported to bind to most H-2 and HLA alleles (Apostolopoulos *et al.*, 1997a; Apostolopoulos *et al.*, 1997b). For example, the 9mer SAPDTRPAP and 8mer SAPDTRPA bind to and are presented by H-2Kb, even though they do not contain the K^b consensus anchor motifs (Phe/Tyr) at position 5/6 and (Leu) at position 8/9. Their binding is such that the N terminus of the peptide is buried in the groove, the middle portion protrudes outwards and the C terminus is free and can react with antibodies specific for the peptides. The high-resolution crystal structure of H-$2K^b$ complexed with the 8mer peptide shows that, by contrast with high affinity H-$2K^b$ binding peptides, this peptide is less buried in the MHC and two water molecules that are expected to occupy vacated or non-optimally filled pockets are missing. This leaves a large cavity in one of peptide binding pockets that contributes to the low affinity of binding (Apostolopoulos *et al.*, 2002).

The general consensus to date is that T lymphocytes are the major mediators of anti-tumor immunity. Based on the fact that majority of tumors are MHC class I positive but lack MHC class II molecules, numerous studies have focused primarily on $CD8^+$ CTLs, which are considered the ultimate effectors at the tumor site. Although CTLs can directly kill tumor cells and their effectiveness *in vivo* has been demonstrated, it has now become evident that long-lasting anti-tumor immunity depends on successful activation of tumor-specific $CD4^+$T helper cells. $CD4^+$T cells can be divided into T helper 1 (Th1) and Th2 cells based on their cytokine secretion profile (Morel and Oriss, 1998). Th1 cells help prime $CD8^+$T cell responses while Th2 cells help establish humoral immune responses. In addition to their role in CTL priming, Th1 cells also secrete cytokines (such as IL-2) required for maintaining $CD8^+$T cell growth and proliferation (Greenberg, 1991; Rosenberg, 1999).

Taking all these observations into consideration, an ideal MUC1 vaccine would be defined as one that induces strong helper and cytolytic MUC1-specific cellular responses, stimulates antibody production and also provides long-term immunologic memory to the tumor. In the attempt to achieve these goals, groups working on the immunobiology of MUC1 have tested numerous antigenic formulations in several animal models. We will discuss below past and current vaccination strategies that have been tested against a variety of tumors in pre-clinical animal models or in clinical trials in patients.

B. MUC1 immunogenicity in animal models

1. Non-human primates

The majority of studies on MUC1 immunogenicity, performed in experimental animals, used wild type mice or rats as recipients of various MUC1 vaccines. Since human MUC1 is xenogeneic to these animals, such studies do not provide a realistic evaluation of its immunogenicity and potential for immunotherapy in humans. We have conducted a number of studies in healthy chimpanzees, a more relevant animal model in which MUC1 is highly homologous to human MUC1 (Barratt-Boyes *et al.*, 1998; Barratt-Boyes *et al.*, 1999; Pecher and Finn, 1996). These studies show that immunization with soluble glycoprotein, or synthetic peptides leads to antibody responses but no CTL. By contrast, Pecher et al., (Pecher and Finn,

1996) showed that when irradiated autologous EBV immortalized B cells transfected with MUC1 cDNA, and expressing tumor-like MUC1 are used as a vaccine they elicit CTL. We have also tested a vaccine based on *in vitro*-derived chimpanzee dendritic cells pulsed with MUC1 peptide and have shown that these can elicit T cell responses after a single intravenous injection (Barratt-Boyes *et al.*, 1998). The lack of a tumor model in chimpanzees did not allow evaluation of the efficacy of these responses in tumor rejection.

Macaques express MUC1 that differs by 5 amino acids out of 20 in each repeat (75% identity) from the human MUC1 VNTR. Using oxidized mannan as a delivery system for 100mer (five repeats) human MUC1 peptide, Vaughan et al (Vaughan *et al.*, 1999) showed that both humoral and cellular anti-MUC1 immune responses could be induced in immunized macaques. The antibody response was predominant, in contrast to responses previously seen in MUC1 in wild type mice where cellular responses were predominant. In a later report, the same group (Vaughan *et al.*, 2000) isolated, sequenced and expressed macaque MUC1. They then vaccinated both mice and macaques with a fusion protein of the monkey MUC1 VNTR conjugated to oxidized mannan. The response elicited by the macaque MUC1 mannan vaccine in two immunized monkeys showed a Th2 type of response, with low titers of anti-MUC1 antibodies of IgG1 and IgM isotypes and no proliferative or CTL responses in the spleen or draining lymph nodes of the immunized animals. The animals remained healthy and showed no signs of autoimmunity throughout the vaccination period. By contrast, wild type mice immunized with mannan-macaque MUC1 (a xenoantigen) exhibited a strong cellular response and protection to tumor challenge, indicative of Th1 (low antibody, strong CTL) immunity. Moreover, mice immunized with self murine muc1 conjugated to mannan (Vaughan *et al.*, 2000) and cancer patients immunized with mannan-conjugated human MUC1, display Th2 responses. These studies reiterate our findings in chimpanzees and show that non-human primates like macaques are more suitable preclinical models, reflecting more closely human responses than the murine model where MUC1 is a xenoantigen. Nevertheless, as with the chimpanzees, this model is expensive and does not allow evaluation of *in vivo* responses to tumor challenge.

2. Murine models

There is only slight homology between the human MUC1 and mouse muc1 molecules. Because of this, any vaccines based on the human MUC1 administered to wild type mice will trigger immune responses that will not reflect the intrinsic immunogenic properties of MUC1 but rather its foreignness. However, mouse models are the only systems in which tumor rejection studies can be performed.

Mice transgenic for human HLA class I molecules represent an attractive model system to study immunogenicity of CTL epitopes and immunodominance in immune responses. While MUC1 immunization of these mice does not circumvent the problems associated with MUC1 seen as "foreign" by the murine immune system, it could lead to identification of MHC class I restricted CTL epitopes with potential for vaccine design.

As discussed above, immune recognition of MUC1 peptide sequences within the tandem repeats can occur in both MHC-restricted (discussed above) as well as MHC-unrestricted manner. We and others (Apostolopoulos *et al.*, 1997b; Domenech *et al.*, 1995) have shown that the STAPPAHGV peptide sequence derived from the VNTR region of MUC1 constitutes a target for both HLA-A11 and HLA-A2-restricted CTLs. HLA-A11 and HLA-A2 are two of the more frequently expressed MHC class I alleles.

Prediction algorithms using computer software show that tandemly repeated 20mer MUC1 sequence generate epitopes that do not fully comply with the classical binding motifs to human MHC class I molecules and are considered to bind in a non-conventional manner (Apostolopoulos *et al.*, 1997b). In contrast, MUC1 regions outside VNTR display many epitopes with potential to bind to HLA-A molecules. The combined results from three research groups (Brossart *et al.*, 1999; Carmon *et al.*, 2000; Heukamp *et al.*, 2001)show that there are six HLA-A2 restricted and naturally processed non-VNTR epitopes identified to date that can generate human CTLs able to recognize MUC1-expressing tumors. Three of these have been tested *in vivo* in HLA-A2/K^b transgenic mice (Heukamp *et al.*, 2001). Mice were injected with peptide in incomplete Freund's adjuvant (IFA) and subsequently challenged with B16 murine melanoma cells transfected with human MUC1 cDNA and the HLA-A2/K^b gene. The tumor-rejection experiments suggested that vaccination with peptides from the N-terminal region of MUC1 that bind HLA-A2 *in vitro*, renders protection against tumor challenge. Similarly, vaccination with dendritic cells pulsed with the same set of peptides leads to protection against tumor growth.

VNTR-derived peptide epitopes could potentially be generated in large numbers by the tumors as well as by normal epithelial and antigen presenting cells. As much as their abundance could be considered an advantage in generating robust anti-tumor responses, it could be also regarded as a strong stimulus for autoimmunity. Epitopes generated from sequences outside the MUC1 VNTR, represent a less abundant source of antigen, at least in normal MUC1-expressing cells. Vaccination with non-VNTR HLA-A2 –restricted MUC1 epitopes might circumvent autoimmunity; however, such an approach could lead to emergence of antigen loss variants of the tumor.

Transgenic (Tg) mouse models expressing human tumor antigens provide a suitable model for testing their immunogenicity given the fact that they are endogenous self proteins to which both B and T cell tolerance should develop. Several lines of human MUC1-transgenic mice have been generated on the C57Bl6 (Rowse *et al.*, 1998), BALB/c (Carr-Brendel *et al.*, 2000) and DBA (Acres *et al.*, 2000) backgrounds. These mice show MUC1 expression with a distribution pattern similar to that in humans and better reflect immunopathology of human tumors in which MUC1 is a self antigen subjected to the mechanisms of immunological tolerance. MUC1 transgenic mice challenged with MUC1-bearing syngeneic tumors fail to develop effective anti-tumor responses (Rowse *et al.*, 1998), in contrast to wild type mice that reject all MUC1 positive tumors. In addition, there is no antibody class switching in Tg mice immunized with MUC1 peptide, suggesting that these animals are tolerant to MUC1 in both the T and B cell compartments.

With the advent of the MUC1 Tg mouse model, we and others have focused our attention on vaccination strategies that could break tolerance and elicit efficient antitumor immune responses. A very important aspect of such immunizations, besides their effectiveness to rejecting tumors, is the possibility of eliciting undesired autoimmune responses during the course of tumor rejection.

Soares et al., (Soares *et al.*, 2001b) have shown that three vaccination protocols based on a peptide composed of seven tandem repeats of MUC1 elicit different immune effectors in the Tg vs. wild type mouse, with different potential for tumor rejection. The vaccines were either MUC1 peptide plus murine GM-CSF as an adjuvant, MUC1 peptide plus the adjuvant SB-AS2, or MUC1 peptide pulsed on DC. Cytokine and antibody production by T and B cells, respectively, ability of mice to reject tumors and signs of autoimmunity were monitored in both Tg and wild type mice vaccinated with either of the three vaccines. The only vaccination

protocol that led to tumor rejection in both types of mice was the peptide-pulsed DC vaccine. In Tg mice the tumor rejection was solely attributed to IFN-γ producing $CD8^+$ T cells. Importantly, although a high degree of tolerance to MUC1 was expected, it was surprising that tolerance in the helper T cell compartment could not be overcome by the DC-based vaccine. We found this to be intriguing, especially since we have shown that helper responses to the same MUC1 peptide can be elicited in immunized healthy chimpanzees (Barratt-Boyes *et al.*, 1999) and that MUC1 loaded onto human DC can prime MUC1-specific $CD4^+$ T cell responses *in vitro* (Hiltbold *et al.*, 1998). However, in a subsequent study, Soares et al., were the first to demonstrate that $CD4^+$ T cell tolerance in MUC1 Tg mice could be overcome upon vaccination with MUC1 peptide microencapsulated in poly d,l-lactic-co-glycoli acid (PLGA), administered in the absence of adjuvant (Soares, 2001). The MUC1 microsphere-based vaccine triggered Th1 helper responses, elicited MUC1-specific CD8+ responses and caused tumor rejection of MUC1-expressing tumors. Importantly, no autoimmune responses in MUC1 expressing tissues were detected in immunized mice.

Administering MUC1 in biodegradable microparticles could result in enhanced immunity through several possible mechanisms. First, the antigen, incorporated within the copolymer matrix, can be delivered in high concentration directly to antigen presenting cells (APC) (such as Langerhans cells in the skin or resident dendritic cells and macrophages in the periphery. These microparticles are taken up via phagocytosis by APC and the internalized particles will release the antigen for processing in the MHC class II pathway. Some of the encapsulated antigen will also enter the cytoplasm where it will be processed for binding to MHC class I molecules. Thus, PLGA microparticles could also elicit both MHC class II-(helper) and I- (cytotoxic) restricted responses. Finally, the polymer coating of the antigen provides a depot-like system for sustained release of MUC1, thus boosting the efficiency of vaccination by increasing the persistence o f antigen.

Mukherjee et al have reported (Mukherjee *et al.*, 2000) a double transgenic mouse model (called MET), that is a result of a cross between MUC1 Tg mice and ET mice bearing an oncogene that causes spontaneous tumors in the pancreas. MET mice express human MUC1 as a self molecule in the pancreas hence their spontaneous pancreatic adenocarcinomas also express MUC1. The animals develop pancreatic pathology that resembles the progression of human pancreatic cancer. Thus, MET mice are valuable tools for preclinical testing of MUC1 vaccines for pancreatic cancer.

For patients with adenocarcinomas of the pancreas the treatment options currently available (pancreatectomy, radiation and chemotherapy) are aggressive, toxic and most of the times inefficient, as they do not decrease the mortality rate. In fact, pancreatic adenocarcinoma is the fourth leading cause of cancer death in the US, with a five-year survival rate of only 3%. Moreover, even for the 15-20 % of patients who are diagnosed early and undergo potentially curative resection, followed by radiation and chemotherapy, the 5-year survival is only 20%, due to persistence of residual tumor cells that can then proliferate at metastatic sites. We and others consider that eliciting effective immune responses that could identify and eliminate the transformed cells left over after surgical resection of the primary tumor, could provide additional help to enhance survival. As with most cancer types, various genetic modifications have been linked to increased incidence of pancreatic adenocarcinomas. Predisposed patients undergo local inflammatory changes that result in local production of growth factors, cytokines and reactive oxygen species. These changes induce cell proliferation that, associated with inherited genomic

instability, leads in time to tumorigenesis. Identifying patients at risk and treating them early with a preventive vaccine could be more beneficial for long term survival.

Most of the mouse models, prior to development of the MET mouse, used direct injections into the pancreas of either tumor cell lines or xenografts of primary human pancreatic tumors. Morikane et al., (Morikane *et al.*, 2001) have shown that it is more difficult to induce immune responses to tumors transplanted to the pancreatic site than to pancreatic tumor cells injected at the subcutaneous site. While $CD8^+$ T cells are required for rejection of Panc02-MUC1 pancreatic tumors at subcutaneous site, both $CD4^+$ and $CD8^+$ T cells are required for rejecting tumors at a pancreatic site, confirming the fact that local environment (cytokines, hormones, expression of MHC complexes and of adhesion molecules) can dramatically influence the outcome of anti-tumor immune responses.

Pancreatic tumors that spontaneously develop in MET mice overexpress underglycosylated MUC1. As the tumors progress the MET mice exhibit, similarly to cancer patients, increased levels of circulating MUC1. Mukherjee et al (Mukherjee *et al.*, 2000) have shown that non-immunized MET mice developed MUC1-specific CTLs that could lyse as much as 80% of MUC1-transfected B16 murine melanoma target cells (compared to only 20% of the control MUC1-negative B16 cells). The killing was MHC class I restricted and one of the peptide epitopes recognized by some of the CTLs matched the epitope identified in humans, the STAPPAHGV from the MUC1 VNTR. Despite the fact that MET mice develop identifiable CTL responses, those CTLs are inefficient in preventing spontaneous growth of tumors in vivo, and 90% of mice die of pancreatic cancer by 16 weeks of age. However, when adoptively transferred in tumor-challenged MUC1 Tg mice, the MUC1 specific CTLs are able to prevent growth of MUC1-expressing B16 tumor cells and not of MUC1 negative control tumors. Evasion of CTLs by the pancreatic tumors in MET mice can be explained by many factors, generally applicable to immune effector cells in a tumor environment: down-regulation of surface MHC I, antigenic modulation, upregulation of FasL, secretion of inhibitory cytokines, low avidity CTLs etc. This proves once again that the presence of CTL responses cannot be used as the sole indicator of clinical outcome and that broader immune responses from helper T cell as well as B cells should also be examined. Furthermore, MET mice with fully developed aggressive pancreatic tumors, might not provide the right setting for therapeutic vaccination. However, we consider (for reasons underlined below) that early MUC1 vaccination of MET mice might provide a more appropriate approach for modeling cancer prevention, rather than therapy.

The pancreae of MET mice contain displastic acinar cells producing underglycosylated MUC1 by 3 weeks of age. *In situ* carcinomas can be diagnosed by week 13 and by 15 weeks the mice grow well- differentiated pancreatic tumors. This progression resembles (on a different time scale) what has been described in humans, and by attempting to vaccinate MET mice early with an efficient and safe vaccine capable of eliciting strong B and T cell responses, one could hope to achieve a robust level of protection to cancer development. However, one should keep in mind, that despite the relatively slow rate of tumor progression seen in MET mice, it could still be too quick to fully allow tumor specific immune responses to develop. Thus, this model may not accurately reflect the kinetics of tumor development seen in cancer patients, who might live with premalignant lesions for long periods of time before developing pancreatic carcinomas, and who might have even a better potential to destroy incipient tumors if properly vaccinated. Consequently, vaccination approaches inducing immune responses capable of slowing down tumor progression in animal models of spontaneous tumor development, should be regarded as

encouraging and should prompt further exploration of such vaccines for cancer prevention in humans.

VI. MUC1 VACCINES

A. Peptide Vaccines

Vaccines based on synthetic peptides have the advantage of being readily available, although they require the identification of exact epitopes recognized by T or B cells. Most peptide vaccines have been tested for their ability to elicit strong CTL responses; however, optimal vaccine formulations should also include one or more antigen-specific T helper epitopes. Helper responses to MUC1 have not been detected to date in cancer patients. Therefore, identification of MUC1-derived helper epitopes and testing such epitopes *in vivo* is of crucial importance and needs to be further addressed.

We conducted one of the first peptide cancer vaccine clinical trials. The vaccine consisted of a 105mer synthetic MUC1 peptide admixed with *Bacillus Calmette-Guerin* (BCG) and was administered to 63 patients with adenocarcinomas of breast, pancreas or colon (Goydos *et al.*, 1996). BCG is an attenuated form of *Mycobacterium bovis* and has well known adjuvant properties. The vaccine was associated with some toxicity that involved skin breakdown at the vaccination site and various degrees of other symptoms such as fever, chills, nausea and vomiting. Skin biopsies at the injection sites showed delayed type hypersensitivity (DTH) reactions to MUC1 peptides and intense T cell infiltration. Seven out of 22 patients tested showed a 2-4 fold increase in MUC1-specific CTL precursor frequency.

BCG can also be genetically engineered to deliver various antigens in humans. Very recently, Chung et al., (Chung *et al.*, 2003) engineered BCG to express a 22 tandem repeat-long MUC1 cDNA and to simultaneously secrete human IL-2. The BCG- hIL-2MUC1 vaccine was able to inhibit tumor growth in SCID mice reconstituted with human peripheral blood lymphocytes (PBLs) and xenografted with the MUC1 positive ZR75-1 human breast cancer line.

Musselli et al., (Musselli *et al.*, 2002) have tested six breast cancer patients vaccinated four times each with a 106mer MUC1 peptide covalently linked to KLH and administered in the presence of QS21 adjuvant. The patients developed strong anti-MUC1 IgM and IgG responses. The IgG antibodies were of the IgG1 and IgG3 isotypes. PBMCs were tested to detect MUC1-specific T cell responses using proliferation assays and ELISPOT assays for IFNγ production. Only sporadic increases in T cell precursors specific for MUC1 were detected. Reddish et al (Reddish *et al.*, 1998), have used 16mer MUC1 peptide conjugated to KLH and administered in the presence of DETOX as an adjuvant to 16 metastatic breast cancer patients. Three patients developed anti-MUC1 IgG responses and seven out of the eleven patients tested developed MHC class I (HLA-A2, A1 and A11) restricted CTLs.

Acres et al., (Acres *et al.*, 2000) have tested the ability of peptide vaccines to elicit MUC1-specific immune responses in MUC1 Tg mice. One of the vaccines was a 100 amino acid-long MUC1 peptide, corresponding to five tandem repeats, expressed in *E. coli* as a fusion protein with glutathione S- transferase (GST). This fusion protein stimulates weak CTL activity (Apostolopoulos *et al.*, 1995b). Coupling of mannan to the GST part of the fusion protein (MFP, also used in clinical trials- (Karanikas *et al.*, 1997)), significantly increased the CTL precursor frequency in immunized MUC1 Tg mice. There were no signs of autoimmunity in vaccinated mice

B. DNA Vaccines

DNA vaccination with a MUC1-encoding plasmid offers yet another method to target antigen to DC and muscle cells for antigen presentation. This method has been reported to induce both cellular and humoral immunity; moreover, due to prolonged expression of the antigen, it also elicits immunological memory.

Graham et al., (Graham *et al.*, 1996) and later Johnen et al., (Johnen *et al.*, 2001) have administered intramuscular immunizations to C57/Bl 6 wild type mice using 50- 100 μg MUC1 cDNA. Mice were then monitored for their ability to reject human MUC1-expressing syngeneic tumors. As expected, approximately 80% of the mice were protected against tumor challenge and both humoral and cellular mediated immune responses were detected. Although the relevance of these findings is limited to the fact that wild type mice develop strong anti- human MUC1 immune responses, which they see as "foreign", they confirmed that injected DNA was expressed and that the animals developed antibody responses to the protein after immunization with DNA alone.

One important aspect of MUC1 DNA vaccination that needs to be considered is the fact that MUC1 produced by *in vivo* transfected muscle cells or APC would be the normal rather than the tumor form. Moreover, MUC1 molecules released from the surface of such expressing cells are heavily glycosylated and not likely to be processed by adjacent DC, due to defective intracellular trafficking (Hiltbold *et al.*, 2000). Furthermore, persistent expression of a self molecule could also lead to a state of autoimmunity or a state of immunological unresponsiveness.

C. Dendritic cell-based vaccines

Loading DC with tumor antigens for presentation to CD4 and CD8 positive T lymphocytes has been employed by many scientists in their approach to induce strong antitumor responses (for review see (Zhou *et al.*, 2002)). DC have the ability to process the antigen intracellularly and to present peptide epitopes to both $CD4^+$ (direct priming) and $CD8^+$ T cells (cross-priming/cross-presentation). DC also have the exclusive ability to prime naïve CD4 and CD8 positive T cells. They express high levels of MHC class I and II complexes, adhesion (CD11a, CD11c, ICAM-1,-2 and -3 etc) and costimulatory molecules CD80 and CD86, as well as molecules regulating costimulation such as CD40. Expression of many of these molecules varies with different stages of DC maturation. For example, adhesion molecules, CD80, CD86 and MHC complexes are upregulated upon maturation, especially following CD40 ligation. DC function is carried out in three interrelated stages: antigen uptake (through various mechanisms), intracellular processing (in either MHC class I or II compartments) and epitope presentation/T cell priming. In peripheral tissues, immature DC are highly endocytic. Following activation and migration from the tissues to lymph nodes they become highly efficient at antigen processing and presentation. Numerous animal models to date have demonstrated the ability of tumor antigen-loaded DC to prime both $CD4^+$ and $CD8^+$ T cells and to confer protection from tumor challenge. DC-based vaccines have been tested lately in cancer patients and encouraging phase I/II results are emerging (Fong and Engleman, 2000).

Several approaches have been used to arm DC with tumor antigens for use in clinical trials (Zhou *et al.*, 2002). DC pulsed with peptides derived from antigens such as MART-1, MAGE-1, CEA and PSA have all been used to elicit in vitro CTL responses. We (Domenech *et al.*, 1995) and others (Apostolopoulos *et al.*, 1997b; Brossart *et al.*, 1999) have shown that DC pulsed with MUC1 peptides able to bind HLA can elicit MUC1-specific CTL responses

restricted by that haplotype. This approach has the advantage of focusing on short peptides (8-11 aminoacids in length) that can be easily synthesized for large scale immunizations. Most importantly, if the peptide epitopes are tumor-specific, the $CD8^+$ CTLs target cancer cells exclusively, thus limiting the possibility of inducing autoimmunity. However, MUC1 has identical peptide sequence in normal and tumor cells. HLA molecules at the basolateral surface of normal MUC1-expressing epithelial cells can present MUC1 peptides. Following immunization, these peptide-MHC complexes could be recognized by "armed" $CD8^+$ effectors, possibly leading to destruction of normal MUC1 positive tissues. In addition, several other major disadvantages of vaccines based on MHC class I peptide-loaded DC, may limit their clinical applications. First, elicitation of significant anti-tumor responses requires T cell help, strong humoral responses as well tumor-specific immunological memory. Thus, peptides vaccines must include longer peptides that can bind to human MHC class II molecules and elicit helper $CD4^+$ restricted T cell responses. Due to the paucity of defined MHC class II-restricted peptides, heterologous proteins have often times replaced tumor-specific helper epitopes. While these proteins can sometimes be effective in eliciting antibodies and CTLs, they do not provide the molecular basis for long-term tumor-specific memory responses. Second, peptides used for DC loading are restricted to specific HLA haplotypes and are only applicable to MHC-matched patients. Given the tremendous variability in HLA haplotypes among cancer patients, it is not feasible to attempt identification of all peptide candidates for vaccine formulations. Third, targeting effector mechanisms to a narrow spectrum of antigenic epitopes could lead to emergence of antigen loss variants of the tumor (Ikeda *et al.*, 1997; Kerkmann-Tucek *et al.*, 1998; Slingluff *et al.*, 2000).

Longer protein tumor antigens are alternatives to short peptides and are considered to have certain advantages. Their use in vaccine formulations does not require identification of specific epitope sequences. Processing of whole proteins by DC generates multiple peptide epitopes presented by the host's MHC that are not restricted to single alleles. Soluble proteins pulsed onto immature DC are endocytosed and processed in the MHC class II pathway leading to presentation of antigenic epitopes to $CD4^+$ T cells thus ensuring elicitation of tumor-specific helper responses. Finally, endocytosed proteins can gain access to the MHC class I pathway where they undergo proteasomal processing for presentation on MHC class I complexes (crosspresentation) (Brossart *et al.*, 1997; Mitchell *et al.*, 1998; Paglia *et al.*, 1996; Porgador *et al.*, 1996).

MUC1 is a tumor antigen that differs quantitatively (due to overexpression) and qualitatively (due to changes in glycosylation) on tumor versus normal cells. If whole MUC1 antigen was to be used for loading onto DC, large amounts of tumor-like MUC1 glycoprotein would be needed for vaccine preparation. Tumor mucin can be obtained from either tumor cells grown *in vitro* or ascites fluid obtained from cancer patients. Purification procedures from tumor cells are labor intensive and generate low amounts of tumor MUC1 glycoprotein. Expression of recombinant MUC1 in *E. coli* results in its rapid degradation by bacterial proteolytic enzymes (Dolby *et al.*, 1999). MUC1 made by insect cells infected with a recombinant baculovirus vector (Soares *et al.*, 2001a) is a good source of tumor-like MUC1 especially since insect cells glycosylate MUC1 only at very low levels. This baculovirus encoded MUC1 (BV-MUC1) is immunogenic in Balb/c mice that generate antibodies that cross-react with human tumors. Due to its very low glycosylation, BV MUC1 has similar immunogenicity to the synthetic 100mer

MUC1 peptide and might not provide additional benefit to this form, currently employed in clinical trials.

The most "authentic" tumor-like MUC1 glycoprotein is MUC1 purified from ascites fluid of cancer patients (ASC-MUC1). Tumor cells release soluble MUC1 which can drain to regional lymph nodes, enter peripheral circulation and be found circulating in patients' sera. Beatty et al (Beatty *et al.*, 2001) have reported the biochemical structure of ASC-MUC1 that was isolated from sera and ascites fluid of patients with late stage breast and pancreatic cancers. This circulating form of MUC1 is of major interest since this form, possibly the only circulating form of MUC1 available to patients' APC (especially DC) *in vivo*, would be expected to be taken up, processed and presented by APC to helper T cells. Hiltbold et al., (Hiltbold *et al.*, 2000) have shown that ASC-MUC1 is effectively endocytosed by DC through mannose receptors. However, following uptake, the protein is retained long-term in early endosomes and is not transported to late endosomes or MHC class II compartments for proteolytic processing. This block in intracellular trafficking of ASC-MUC1 and its inefficient processing in the MHC class II pathway represents a specific mechanism by which cancer patients' T cells are kept unresponsive to this tumor antigen. Interestingly, long-term retention in early endosomes of the internalized Ag by DC (which does not interfere with their ability to process and present other antigens) was also observed for another glycoprotein tumor antigen, Her-2/neu, which also fails to elicit Th responses in vivo. Understanding how whole protein antigens are handled by DC and how their structure influences processing and presentation to immune effectors is critical for any effort directed toward manipulating the immune response against those antigens and an important consideration in the design of cancer vaccines. The obstacle in intracellular processing of ASC-MUC1 by DC can be overcome by MUC1 peptide vaccines that present DC with the form of MUC1 that can be efficiently processed. This vaccine is currently in clinical trials.

In addition to developing MUC1 peptide vaccines, we have more recently focused on the immunogenic properties of MUC1 glycopeptides. MUC1 made by normal cells is highly glycosylated with branched O-linked oligosaccharides (Gendler and Spicer, 1995). By contrast, in tumor cells, MUC1 O-glycosylation is prematurely terminated, leading to the accumulation of short carbohydrate precursors such as the monosaccharide Tn (GalNAcα1-O-S/T) or disaccharide T (Galβ1-3GalNAcα1-O-S/T), and their sialylated forms sTn and sT, respectively (reviewed in (Baldus and Hanisch, 2000). These tumor-specific carbohydrates are O-linked to serines and threonines in the tandem repeat domain of the MUC1 molecule.

Using synthetic MUC1 glycopeptides bearing tumor-like Tn and T saccharide epitopes, we addressed the question whether such saccharide residues are removed during antigen processing by DC when exogenously fed MUC1 glycoproteins and whether DC are able to generate glycopeptide epitopes for presentation on MHC class II. Our results show (Vlad *et al.*, 2002) that DC endocytose glycopeptides, transport them to acidic compartments, process them into smaller peptides, and present them on MHC class II molecules without removing the carbohydrates. Glycopeptides that are presented on DC are recognized by T cells, suggesting that a much broader repertoire of T cells could be elicited against MUC1 than expected based solely on peptide sequences. Further *in vivo* studies, aimed at measuring the efficiency of MUC1 glycopeptide-specific T cell responses against tumors, need to be performed.

An alternative method of providing DC with whole tumor antigens is by creating DC-tumor cell hybrids, such as fusions of DC with MUC1-expressing carcinoma cells. The fused

cells express MHC class I and II and costimulatory molecules, normally present on DC. In addition, unlike DC, the hybrid cells also display MUC1 (and possibly other tumor-specific antigens) on their cell surface. Upon injection, these hybrids migrate to draining lymph nodes where they distribute to T cell areas in a manner similar to normal DC (Koido *et al.*, 2002). As shown by Gong et al, (Gong *et al.*, 1998), the hybrid cells are able to break T cell tolerance to MUC1 in MUC1 Tg mice and to trigger CTLs that are able to reject MUC1-expressing pulmonary metastases (Gong *et al.*, 1998).

Immunization with DC-tumor cell hybrid vaccines has the potential of eliciting unwanted autoimmunity. No autoimmunity was detected in vaccinated MUC1 Tg mice. However, the preclinical mouse MUC1 Tg model displays an unexpectedly high tolerance to human MUC1 and may not parallel the clinical situation. Breaking tolerance in humans might require a less intense immune response; therefore, lack of autoimmunity in mice after immunization with DC-tumor hybrids should be carefully considered before translating it into clinical practice.

Induction of antitumor immunity with DC transfected with tumor antigen-encoding RNA represents another approach for DC-based vaccines. This approach, pioneered in Eli Gilboa's laboratory (Boczkowski *et al.*, 1996), is based on the findings that vaccination of mice with DC pulsed with RNA from OVA-expressing tumor cells confers protection against challenge with OVA-expressing tumors. Moreover, monocyte-derived human DC transfected with RNA encoding the CEA tumor antigen (Gilboa *et al.*, 1998; Nair *et al.*, 1998) stimulate potent CEA-specific CTL responses in vitro. Similarly, Koido et al., (Koido *et al.*, 2000), showed that vaccination of wild type mice with MUC1 RNA-transfected DC elicits MUC1-specific CTL responses, resulting in rejection of MUC1-tansfected MC38 tumor cells but not of untransfected cells. The same immunization protocol, effective in wild type mice, failed to protect MUC1 Tg mice from tumor challenge. However, co-administration of IL-12 with RNA-transfected DC resulted in increased MUC1-specific CTL activity and rejection of MUC1/MC38 tumors.

MUC1 expression by DC can by achieved by gene transduction using MUC1-encoding recombinant adenovirus. DC endogenously expressing MUC1 present MHC class I-restricted MUC1 peptides that could trigger naïve $CD8^+$ T lymphocytes. Maruyama et al. (Maruyama *et al.*, 2001) have shown that, despite the efficiency seen in murine systems, adenoviral gene transduction of human DC has limited efficiency, with only 39% of transduced DC showing MUC1 protein expression. Nevertheless, the transduced DC were able to induce MUC1 specific CTLs *in vitro*, although not better than peptide-pulsed DC. This approach has the same problem as DNA because normal MUC1 is being made.

Despite the fact that DC vaccines have shown encouraging results, the strategy of using *in vitro*-generated DC from each cancer patient for reinjection after antigen loading has major technical limitations. All experimental steps performed *ex vivo* (culture of large numbers of precursors in the presence of cytokines, purification of immature DC, loading of DC with desired antigen) are costly, time consuming and pose the risk of contamination. As an alternative approach, *in vivo* loading of DC using antigenic formulations with increased uptake efficiency (like liposome-delivered tumor peptides (Ludewig *et al.*, 2000), or injections of Fas- expressing apoptosing autologous tumor cells (Chattergoon *et al.*, 2000)) eliminates the need for *ex vivo* manipulation and could be more applicable on a larger scale.

D. Recombinant vaccinia virus vaccines

Immunization of mice (Acres *et al.*, 1993) or rats (Hareuveni *et al.*, 1990) with a recombinant vaccinia virus that expresses MUC1 upon infection of mammalian cells protects animals against challenge with MUC1 expressing but not control, MUC1 negative syngeneic tumor cells. The vaccinia genome is subjected to high-frequency homologous recombination and, therefore, has unstable expression of the tandem repeats. Akagi et al (Akagi *et al.*, 1997) have generated a recombinant vaccinia virus containing a modified "mini" MUC1 gene containing only 10 tandem repeat sequences to minimize vaccinia-mediated rearrangement. The construct was used in combination with recombinant vaccinia virus containing the gene for the murine T-cell costimulatory molecule B7-1. Vaccine efficacy was tested in a MUC1-expressing pulmonary metastases prevention model, and showed that mice inoculated two times with vaccinia-encoded MUC1 were protected from the establishment of metastases. Recombinant constructs that express both MUC1 and IL-2 (Balloul *et al.*, 1994) were also successful in generating detectable CTL responses and blocking tumor growth upon challenge. This later construct has been used (Scholl *et al.*, 2000), together with two other peptide-based vaccines (Acres *et al.*, 2000), to immunize MUC1 Tg mice.

VII. MUC1 VACCINES IN CLINICAL TRIALS

Vaccines based on MUC1 are currently tested in cancer patients with advanced tumors for therapeutic purposes. During the last years, advances in basic immunology and biotechnology have contributed to the design of vaccines with better immunogenic properties. Development of MUC1 antigens with increased immunogenicity, discovery of more potent adjuvants and design of efficient antigen delivery systems are all important contributions for clinical applications of MUC1 vaccines. Some of these contributions and their impact on patients in clinical trials will be mentioned in the section below.

Various MUC1 phase 1 clinical trials have been performed during past several years in two joint medical centers in Victoria and Queensland, Australia. The first vaccine formulation employed by McKenzie et al was a MUC1 peptide coupled to diphtheria toxin. MUC1 antigen comprising five MUC1 tandem repeats was linked to a GST fusion protein and administered with oxidized mannan. In its oxidized form, mannan targets antigen to the mannose receptors on the surface of APC and favors antigen processing in the MHC class I pathway and cross-presentation to CTLs, as shown in preclinical studies on mice (Apostolopoulos *et al.*, 1995a; Apostolopoulos *et al.*, 1995b). By contrast, MUC1 conjugated to reduced mannan favors antigen presentation in the MHC class II pathway and elicits primarily humoral responses in vaccinated mice.

Efficiency of this oxidized mannan-MUC1 GST fusion protein vaccine was tested on 25 patients with metastatic carcinomas of the breast, colon, stomach or rectum (Karanikas *et al.*, 1997). The patients received increasing doses of antigen, administered subcutaneously. Contrary to the expectation that such a vaccine formulation would favor cellular rather than antibody responses, as seen in vaccinated wild type mice, patients immunized to oxidized mannan-MUC1 fusion protein generated strong antibody responses and only moderate cellular proliferative and cytotoxic responses. These results parallel the findings (discussed above) in monkeys vaccinated with either human or macaque MUC1 conjugated to mannan.

More recently (Karanikas *et al.*, 2001), the same investigators tested efficacy of mannan-MUC1 vaccine when administered intraperitoneally in the presence of cyclophosphamide. The intraperitoneal route was chosen based on findings in mice showing better CTL responses to this route of injection. Similarly, the addition of cyclophosphamide increased the frequency of CTL precursors (Apostolopoulos *et al.*, 1998). Of 41 patients with adenocarcinomas of the breast, colon, stomach, rectum, prostate and ovaries, 60% developed high titer antibody responses of IgG1 isotype and only 28% developed cellular responses, as detected by proliferation and cytotoxicity assays or intracellular detection of TNF-α and IFN-γ in response to MUC1 stimulation. Cytotoxic T lymphocytes detected in 20% of the patients were of low frequency and potency. While the route of administration made a difference, with antibody titers 10 fold higher following intraperitoneal (versus intramuscular) vaccination, the presence of cyclophosphamide failed to skew the immune response from humoral towards cellular immunity.

In summary, MUC1 linked to GST fusion protein and delivered with oxidized mannan could be considered potent for antibody production but of limited efficiency for the induction of cellular antitumor responses. The vaccine is generally well tolerated with the only toxic effect being erythema at the injection site.

Scientists from Sloan Kettering Cancer Center in New York have tested MUC1 peptide vaccines in various clinical trials. According to preclinical studies, covalent attachment of MUC1 (or of other cancer antigens like gangliosides GD3 and GM2) to KLH plus the use of a potent immunological adjuvant showed efficient induction of antibody responses (Zhang *et al.*, 1996) (Livingston, 1995). Out of 19 different adjuvants tested by Kim et al in mice, QS-21, a natural saponin, exhibited the best adjuvant properties (Kim *et al.*, 1999). Furthermore, (Kim *et al.*, 2000) combinations of QS-21 with several other adjuvants (of different chemical structures) resulted in significant increases in anti-MUC1 antibody titers compared to QS-21 alone. A newly identified adjuvant, GPI-0100, a semi-synthetic saponin adjuvant (Marciani *et al.*, 2000), was at least as potent as any of the adjuvant combinations and significantly more potent than QS-21 alone. Subsequent to their studies in mice, Livingston et al tested the MUC1-KLH plus QS-21 vaccine in patients. In 2000, Gilewski et al. (Gilewski *et al.*, 2000) reported the first results of subcutaneous immunization of nine patients with a history of breast cancer but without evidence of disease, using 30mer MUC1-KLH conjugate plus QS-21 as an adjuvant. Combination of antigen-KLH and QS-21 was effective in inducing antibody responses against other antigens, as seen with gangliosides KLH conjugates that trigger high antibody responses in vaccinated melanoma patients (Livingston *et al.*, 1994; Livingston and Ragupathi, 1997). The 30mer (1.5 repeat) MUC1 peptide vaccine plus QS-21 was well tolerated and resulted, as expected, in significant production of both IgM and IgG antibodies against synthetic MUC1. No T lymphocyte responses were detected. The anti-MUC1 IgM antibodies isolated from seven out of nine patients were able to stain MUC1 on surface of MCF-7 tumor cells, but only minimal staining was observed with the IgG. The epitope recognized by these antibodies lies within the APDTRPA sequence. Within the 30mer MUC1 peptide used as an immunogen, the APDTRPA eptitope was located in both a medial and C-terminal position, with the antibodies being able to "see" the epitope only when located at C terminus. The authors have hypothesized that modest cell surface antibody reactivity (despite the high titers of anti-MUC1 IgGs), could be attributed to dependency on epitope conformation and that the original epitope in the synthetic immunogen may differ from the ones encountered on natural, surface-bound MUC1. In a later report, Musselli et al. showed (Musselli *et al.*, 2002) that 106mer MUC1 conjugated to KLH and

administered with QS-21 triggered IgM and IgG. The IgG antibodies were of IgG1 and IgG 3 isotypes.

In 1999, Brossart et al (Brossart *et al.*, 1999) reported the identification of two immunogenic peptides derived from MUC1 and restricted to HLA-A2. While one of the epitopes (M1.1 peptide) was derived from the tandem repeat region of the protein, the second epitope (LLLLTVLTV identified as M1.2) is derived from the signal sequence of the protein, demonstrating that immunogenicity of MUC1 is not limited to responses against epitopes within the tandem repeats. Also of interest is the fact that M1.1 peptide is a 9mer whose sequence (STAPPVHNV) differs from the classical STAPPAHGV by two amino acids: V in position 6 and N in position 8. These substitutions occur naturally on tumor MUC1 (Brossart *et al.*, 1999) and the epitopes can be presented by HLA-A2 on tumors and antigen presenting cells. Moreover, presence of valine at position 6 increases the binding of the M1.1 peptide to the HLA-A2 molecule.

CTLs induced by *in vitro* priming using autologous DC pulsed with the M1.1 and M1.2 peptides can efficiently lyse target cells pulsed with the peptides or HLA-A2 positive tumor cells (Brossart *et al.*, 1999). Based on this finding, Brossart et al (Brossart *et al.*, 2000) have administered the peptide-pulsed DC vaccine to 10 patients with advanced breast and ovarian cancers, enrolled in a phase I/II study at the University of Tubingen Medical Center. Out of 10 patients, 6 received DC pulsed with HER-2-neu-derived peptides and 4 received MUC1 peptide-pulsed DC, according to the expression of the tumor marker on the tumor. Pre-pulsed DC were subcutaneously injected close to the inguinal lymph nodes, every 14 days for the first six weeks and repeated afterward every 28 days. The authors showed peptide-specific CTL activity detected in the peripheral blood of five of the vaccinated patients, after 3 rounds of vaccinations. Interestingly, one patient treated with MUC1-pulsed DC, also developed MAGE-3- and CEA-peptide-specific $CD8^+$ T cells, while another patient developed MUC1-specific CTLs after vaccination with Her-2/neu peptide-pulsed DC, suggestive of epitope spreading. Induction of CTLs *in vivo* can lead to destruction of tumor cells which are then taken up by APC like DC. Their tumor-derived antigens can then be processed inside the DC and cross-presented to MHC class I-restricted and tumor antigen-specific $CD8^+$ T cells. Overall, these findings show that cancer patients diagnosed with advanced tumors and pretreated with high doses chemotherapy, could still mount efficient antigen-specific cellular responses following vaccinations with peptide-pulsed DC and that such a vaccine is well tolerated.

Brossart et al (Brossart *et al.*, 2001) have recently shown that the list of MUC1-expressing malignancies, comprising mostly epithelial solid tumors and (as previously shown) multiple myelomas, could be extended to other hematopoietic malignancies like acute myeloid leukemia (AML- showing positivity for MUC1 in 67% of cases), follicular lymphoma, chronic lymphocytic leukemia (CLLs) and hairy cell leukemia, all showing varying degrees of MUC1 expression. Using DC derived from PBMCs from normal HLA-A2 positive donors, pulsed with M1.1 and M1.2 MUC1 peptides, the authors have shown that in vitro primed CTLs were able to recognize, in a HLA-A2 restricted manner, primary leukemic blasts as well as multiple myeloma cells and several other AML cell lines endogenously expressing MUC1 protein. Expanding vaccination with MUC1 to patients with hematological malignancies needs to be further explored.

Immunizations of mice using a recombinant vaccinia virus (which encodes for human MUC1 as well as for IL-2) can stimulate CTL responses at levels protective against tumor challenge (Acres *et al.*, 1993). Efficacy of the vaccine was later tested on nine patients with advanced breast cancer in a phase 1 and 2 clinical trial conducted in France. The patients, diagnosed with MUC1 positive advanced breast tumors with chest wall recurrences, were injected intramuscularly in the deltoid muscle with recombinant viral suspension. The vaccination resulted in no significant systemic adverse effects. However, the authors reported only modest MUC1-specific immune responses in vaccinated patients. None of the vaccine recipients were able to mount MUC1-specific antibody responses, as detected by ELISA testing of patients' sera using a MUC1 peptide from the tandem repeat sequence. Based on our experience using MUC1-encoding vaccinia vectors (Bu *et al.*, 1993), it is probable that the lack of antibodies to the MUC1 tandem repeats is due to the fact that vaccinia is recombining out the tandem repeats of the molecule, thus preventing humoral responses against this region. Two of the nine recipients showed MUC1-specific CTL activity against Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines established from each tested patient. The lytic activity was specific against the EBV B cells previously infected with MUC1-encoding vaccinia virus and not against the cells infected with control vaccinia vector.

Vaccinia viral vectors display several advantages that make them attractive vaccine candidates: ability to incorporate large amounts of foreign DNA, wide host cell range, stability, enhanced immunogenicity due to live attenuated vaccinia virus etc. However, there are also disadvantages and they are mostly related to the potential toxicity triggered in patients by the extremely immunogenic vaccinia viral proteins. In addition, we consider that although the stability of the encoded protein may not be a problem for many other antigens, it should be carefully addressed when using MUC1-encoding vaccinia vectors. The MUC1 extracellular domain comprises a large number of tandem repeats with each repeat displaying immunodominant epitopes. Thus, the inability of the virus to preserve transcription of the repeats may result in decreased immunogenicity of the vaccine. Lastly, vaccinia infected cells produce normal forms of MUC1 that might, as discussed above, elicit immunity to normal rather than tumor tissues expressing MUC1.

The quest for the best method of genetically engineering DC to express MUC1 is continuing, as scientists try to identify the most efficient transfection method that is also easy to handle and applicable for use in clinical trials. Recently, Pecher et al, (Pecher *et al.*, 2002) tested a DC-based vaccine in a phase I/II clinical trial using autologous DC transfected with MUC1 cDNA using liposomes. A group of 10 patients with advanced breast, pancreatic or papillary cancer received 2 or 3 subcutaneous immunizations with transfected DC. Efficiency of transfection varied widely with as little as 2% and as high as 53% of DC showing MUC1 expression. While the study demonstrated the feasibility and safety of this vaccination approach, weak immune responses were seen in only 4 patients, of which only one showed stable disease for 3 months after starting vaccination. The other 9 patients showed clinical progression of the disease. Although liposomal transfection is technically easy to execute, it shows high variability in transfection efficiency, and might not be suitable for large scale clinical trials.

One important aspect when considering vaccines based on DC that are genetically manipulated to express MUC1, (following either retroviral, adenoviral or simply naked DNA vaccination), is the nature of MUC1 protein that will be produced. As we have shown (Henderson *et al.*, 1996), MUC1 on DC is fully glycosylated and will not exhibit any tumor-like

(such as Tn or T) epitopes. DC could still prime CTLs to naked peptides generated from the normal processing in the MHC class I pathway. However, transfected cells generate fully glycosylated, normal MUC1 and possibly become, upon uptake by adjacent APC a source of large and heavily glycosylated MUC1 glycoprotein that is difficult to process. Thus genetic manipulation for MUC1 production should ensure production of a MUC1 form that is tumor-like and therefore more suitable for vaccination.

Hybrid cells created by DC-tumor cell fusions provide a tumor-like source of MUC1 and efficient costimulatory properties. Using electrofusion techniques Kugler et al (Kugler *et al.*, 2000) have generated hybrids of allogeneic DC and autologous tumors from 17 patients with metastatic renal cell carcinoma. Following electrofusion (from a total of 50 million DC and 50 million tumor cells) and irradiation, the cells were immediately injected subcutaneously in close proximity to inguinal lymph nodes. All patients had their tumors removed prior to vaccination. Immune responses against MUC1 (expressed on 82% of the primary tumors included) and Her2/neu were used to monitor induction of antitumor immunity. Out of the seventeen patients tested, four had complete tumor remissions, two underwent partial remissions and one had a 'mixed ' response. Additionally, two patients with multiple lung metastatic lesions were stabilized for 17 and 15 months, respectively. Three of the four patients with complete remissions successfully rejected metastases of the lung, bones, lymph nodes and soft tissues. Eight out of 17 patients showed progressive disease. The vaccine was well tolerated and there were no clinical signs of autoimmunity. Despite its partial success, hybrid cells-based vaccines are difficult to generate. Availability of the primary tumor, *in vitro* processing of tumor cells for reinjection, generation of allogeneic DC of different haplotypes to avoid predominance of allogeneic responses, low efficiency of fusion (only 10%), risk of contamination during *in vitro* manipulation and high cost, are all technical factors that limit applicability of this vaccine for large scale immunization.

VIII. CONCLUSIONS AND FUTURE PERSPECTIVES

Important developments in MUC1 research have provided new insights to our understanding of how MUC1 glycoprotein changes during premalignant and later malignant transformation and how these changes are recognized by various immune effector mechanisms. It has been demonstrated by us and others that MUC1 is antigenic: cancer patients with MUC1 positive tumors generate anti-MUC1 antibodies and MUC1-specific CTLs. However, in a tumor environment, these immune responses are inefficient at controlling tumor growth. The aim of current studies by several research groups is, therefore, to potentiate existing antitumor responses against autologous cancer cells, using MUC1 vaccines. The mechanisms that form the basis of immunotherapeutic MUC1 vaccines are underlined in Figure 1. MUC1 antigens can be administered in a variety of forms: as soluble peptides or proteins in the presence of adjuvants, coated on beads, as MUC1-encoding cDNA, via preloaded DC or on DC-tumor cell hybrids. Following vaccination, MUC1-specific T and B cellular immune responses are triggered in secondary lymphoid organs. The armed effectors need to migrate to the tumor site where they are expected to induce anti-tumor defense mechanisms. Ideally, successful vaccination should elicit IgG antibodies, strong helper and cytotoxic responses and long-lasting antitumor immunity.

Major efforts have been made towards the design of better MUC1 vaccines, e.g. synthesis of MUC1 peptides and glycopeptides with improved immunogenic properties, discovery of more potent adjuvants and efficient delivery systems have advanced the field of MUC1 vaccination. Vaccines that showed promising results in preclinical models were later tested on patients in phase I/II clinical trials. However, none of the vaccines tested to date show an ability to successfully trigger both humoral and cellular MUC1 specific responses and to reverse the clinical course of disease in cancer patients. Translating encouraging results from mouse models to patients oftentimes leads to disappointing results, partly due to the fact that patients enrolled in clinical trials usually have large, well established and/or disseminated tumors. These late-stage patients, with impaired immunity and large tumor burdens might display insurmountable barriers to activating immune effector mechanisms.

Many important aspects of MUC1 vaccination still need to be addressed, such as identifying the optimal dose of antigen and optimal frequency of vaccination in order to create a therapeutic window of antigen concentration able to induce efficient T and B cell responses. In addition, we need to identify what anti-MUC1 antibody titers are associated with antitumor effects and what parameters better define the right type of T lymphocyte responses. CTLs, most studied to date, are only one of many players in the anti-tumor response and induction of cytotoxic responses is essential but not sufficient, to control tumor progression. In addition to chemo-and radioresistance, tumor cells can evade CTLs through various mechanisms such as release of inhibitory cytokines or developing resistance to perforin (Lehmann *et al.*, 2000) or to the granzyme B pathway (Medema *et al.*, 2001). In this context, we need to further explore the functional interactions between the immune effectors and the tumor and its microenvironment. Thus, vaccination approaches that combine potent immunogens with modulators of tumor cell resistance to killing, could prove to be beneficial.

Successful vaccine development also depends on our ability to detect immune responses. Currently, a variety of options exist for monitoring MUC1-specific immunity and each technique has its own set of advantages and disadvantages (serological techniques for antibody responses or DTH reactions, ELISPOT assays, tetramer staining, cytotoxic assays etc., for T cell function). However, the challenge still remains to develop immune monitoring systems that are highly reproducible, show enhanced sensitivity and specificity, and better correlate with clinical outcome.

Many of the experimental systems (i.e. mice challenged with tumor cells following vaccination) show that immunity can be activated to prevent tumors. Thus, we can focus in the future on strategies using MUC1 for prevention rather than therapy. Individuals with high genetic risk of developing cancer, or patients diagnosed with MUC1 positive preneoplastic lesions, have an immune system that is not yet suppressed by the tumor and are likely to mount stronger, protective responses upon vaccination. Similarly, vaccination after surgical resection or in the presence of minimal residual disease following standard therapy, might show a better rate of success in preventing tumor recurrence.

We provided here a broad view of MUC1 immunobiology and its impact on cancer vaccine research. MUC1 vaccines can prevent tumors in animal models and are safe to test in cancer patients. The creation of the MUC1 Tg mouse model will further advance our efforts to design better vaccines, able to break immune tolerance, protect against tumors and avoid unwanted autoimmunity. However, the real challenge for MUC1 immunologists in the future will be to decide whether to continue to test these cancer vaccines for therapeutic purposes in

cancer patients, or to begin testing them for their ability to prevent cancer development in patients with premalignant lesions.

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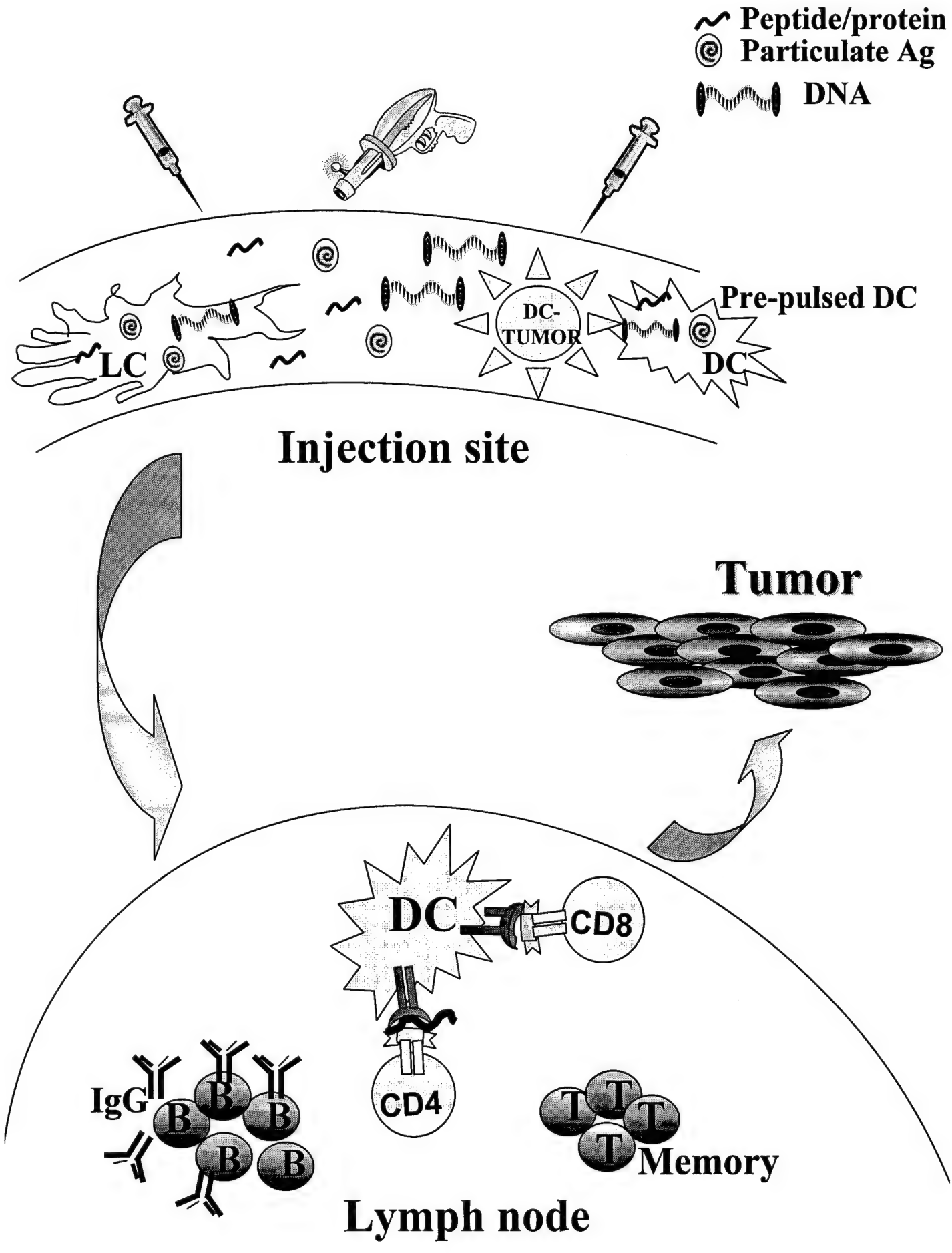
Peptide/protein
Particulate Ag
DNA
LC
DC-
TUMOR
DC
Pre-pulsed DC
Injection site
Tumor
DC
CD8
CD4
IgG
B
T
Memory
Lymph node

FIGURE LEGENDS

Fig.1. Vaccination strategies using MUC1 tumor antigen. MUC1 antigens can be administered as soluble peptides or proteins in the presence of adjuvants, coated on beads, as MUC1-encoding cDNA, via preloaded DC or on DC-tumor cell hybrids. Antigen presenting cells such as dendritic cells (DC) process the MUC1 antigen and present MUC1-derived peptides on MHC class I and II molecules to $CD8^+$ and $CD4^+$ T cells, respectively. Following antigen recognition, MUC1-specific T and B cellular immune responses are triggered and expanded in secondary lymphoid organs. The activated effector cells then migrate to the tumor site where they are expected to induce anti-tumor responses. Ideally, successful vaccination should elicit specific antibodies, strong helper and cytotoxic responses and long-lasting immune memory.

Intratumoral Administration of Ad-CD40L or Dendritic Cells Transduced with Ad-CD40L Induces Antitumor Immunity

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Abstract

The interaction between CD40 and its ligand, CD40L (CD154), plays an important role in the development of both humoral and cell-mediated immunity. Here we have evaluated the ability of local, adenoviral gene transfer of CD40L to elicit an antitumor immune response to established tumors in mice. A recombinant adenovirus encoding the murine CD40L gene (Ad-CD40L) was constructed and tested in murine MC38 colon and TS/A breast adenocarcinoma therapy models. Intratumoral administration of Ad-CD40L resulted in a significant inhibition of MC38 tumor growth when compared with control groups treated with either saline or control adenovirus. In contrast, intratumoral injection of Ad-CD40L did not result in a significant inhibition of TS/A tumor progression. However, a single intratumoral injection of Ad-CD40L transduced dendritic cells (DC) resulted in a significant inhibition of MC38 tumor growth, whereas similar treatment of TS/A tumors induced a complete tumor rejection with systemic antitumor immunity. Taken together, these results demonstrate that intratumoral injection of Ad-CD40L and, in particular, CD40L-transduced DC is an effective approach for inducing antitumor immunity.

Introduction

CD154 (CD40 ligand, CD40L), an important co-stimulatory molecule primarily expressed on activated CD4+ helper T cells (1), is a 33-36 kD type II integral membrane glycoprotein from the TNF superfamily. The receptor for CD154 is CD40, which is expressed on a variety of cell types. In hematopoietic cells, CD40 is present on CD34+ hematopoietic progenitors, B cell progenitors, mature B lymphocytes, plasma cells, monocytes, dendritic cells (DC), eosinophils, basophils, and subpopulations of T lymphocytes. CD40 is also expressed on non-hematopoietic cells including endothelial cells, fibroblasts, and epithelial cells (1). The interaction between CD40 and CD40L is critical for the generation of cell-mediated immune responses (2-4), including antitumor immunity. For example, mice vaccinated with a CD40L expressing plasmid did not develop metastatic tumors following challenge with a lethal dose of tumor cells (5). In addition, it was observed that treatment with anti-CD154 monoclonal antibody inhibited the generation of protective immune responses after the administration of three potent tumor vaccines: irradiated MCA 105 cells, MCA 105 cells admixed with *Corynebacterium parvum* adjuvant, and irradiated B16 melanoma cells transduced with the gene encoding granulocyte macrophage colony-stimulating factor (GM-CSF) (6). Further confirmation of the role of CD40/CD154 interactions in tumor immunity was provided by the overt tumor susceptibility of mice deficient for the CD40 receptor (6).

Professional antigen-presenting cells (APC), in particular dendritic cells (DC), play a central role in the induction of T cell and T-dependent immune responses. Ligation of CD40 expressed by DC provides the signals required by the APCs for initiation of adaptive immune responses. In particular, CD40 mediates an important signaling for the maturation and function of DC both *in vitro* and *in vivo*. Furthermore, CD40 ligation also has been shown to inhibit both

spontaneous (7), and tumor-induced apoptosis of DC (8), and protects B cells from apoptotic death by up-regulating the apoptosis inhibitory protein Bcl-x_L (9). CD4+ T cells stimulate DC through a CD40-CD40L interaction, resulting in DC that can effectively stimulate CD8+ T cells responses (10, 11). In addition, gene transfer of CD40L to DC appears to stimulate a CD8+ response in the absence of CD4+ T cells (12).

The interaction between tumor cells and DC is critical for the regulation of an antitumor response. Based on their unique ability to stimulate T cells, DC have been used to stimulate antitumor immunity in both preclinical models and clinical trials. Administration of DC pulsed with tumor antigens or tumor lysates was able to stimulate specific antitumor responses (13). Moreover, genetic modification of DC to express tumor antigens also resulted in effective immunization following inoculation into mice (14). Alternatively, the injection of untreated DC directly into the tumor mass where DC can acquire tumor antigen followed by migration to lymph nodes or spleen to initiate tumor immunity also has been shown to be an effective strategy (15). The advantage of this approach is that it circumvents the needs for tumor-associated antigens. Thus we have been examining the ability of adenoviral-mediated transfer of genes encoding immuno-stimulatory cytokines to DC to stimulate antitumor response following intratumoral injection. Adenoviral mediated gene transfer of IL-12, IL-18 or GM-CSF to DC resulted in increased antitumor responses following intratumoral injection of modified DC (16). However, it is still not clear which is the most effective approach for enhancing the ability of DC to stimulate systemic immunity following intratumoral injection.

Given the ability of CD40L to stimulate immunity *in vivo*, we were interested in determining the antitumor effects of intratumoral delivery of CD40L. Previously we have demonstrated that genetic modification of tumor cells to express CD40L by retroviral infection

resulted in an effective anti-tumor response during tumor establishment (17, 18). Moreover, part of the antitumor effect of CD40L on tumor cells appeared to be mediated through the inhibition of apoptosis of tumor-infiltrating DC conferred though up-regulation of Bcl-X_L (8). Here we have examined the antitumor effects of CD40L following two different routes of administration in two different murine tumor therapy models. We demonstrate that direct injection of adenoviral vector encoding CD40L (Ad-CD40L) into established tumors resulted in a significant inhibition of the tumor growth in both murine colon MC38 and breast TS/A adenocarcinoma models. In addition, DC transduced with Ad-CD40L and administered to the tumor site induced a strong specific antitumor response. Intratumoral injection of DC/CD40L resulted in a more effective antitumor response than injection of DC engineered to overexpress IL-12. Taken together, these experiments suggest that intratumoral delivery of CD40L by adenovirus-mediated gene transfer or by genetically engineered DC is a highly effective approach to confer antitumor immunity.

Materials and Methods

Tumor cell lines. MC38 colon and TS/A and 4-T.1 breast adenocarcinoma cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine.

Animals. Female 6-8 week-old C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in groups of five in a 12:12 hour light:dark cycle with standard mice chow and water *as libitum*. All animals were acclimatized at least 2 weeks prior to experimentations.

Tumor therapy models. $7.5x10^4$ MC38 cells or $2.5x10^5$ TS/A or 4-T.1 cells were injected subcutaneously in the right flank of mice. On day 7 when the tumor size reached 25-30 mm^2, intratumoral injection of 10^9 pfu of either control adenoviral vectors Ad-ψ5 or Ad-LacZ,

or Ad-CD40L or DC (10^6 cells) non-transduced or transduced with Ad-CD40L was performed in a 100 µl volume. Injection of HBSS served as an additional control. The size of each tumor was measured 3 times weekly with caliper and recorded as tumor area (mm^2). For some studies, where indicated, mice were injected with tumor cells in both flanks.

Preparation of Adenovectors. Ad-CD40L was constructed, propagated, and titered according to the standard protocol previously described (19). Briefly, the murine CD40L cDNA (Immunex Corporation, Seattle, WA), was amplified by PCR using a set of primers to create a SalI site at the 5' end and a NotI site at the 3' end. Then plasmids were digested by SalI and NotI restriction enzymes to release the CD40L cDNA, which was subcloned into SalI-NotI site of the adenovirus shuttle plasmid (pAdlox) to generate pAdlox/mCD40L. In this vector, the inserted cDNA sequence is expressed under the transcriptional control of the cytomegalovirus promoter. The plasmid was linearized with SfiI and co-transfected with the Ad-ψ5-derived, E1, E3 deleted adenoviral backbone, into 293 cells by calcium phosphate precipitation. The recombinant Ad-CD40L vector was isolated from a single plaque, expended in CRE8 cells, and purified over double cesium chloride gradient ultracentrifugation. The purified virus was extensively dialyzed against 10 mM Tris/1 mM $MgCl_2$ sterile viral buffer at 4°C, stored in aliquots at –80°C, and titered on CRE8 cells for plaque forming units (pfu). The generation of Ad-IL-12, Ad-LacZ, Ad-ψ5 and Ad-EGFP (enhanced green fluorescent protein) have been previously described (20).

DC generation. DC were generated from mouse bone marrow precursors as previously described (21). Briefly, femur and tibia marrow cells from C57BL/6 or Balb/c mice were depleted of erythrocytes, T and B lymphocytes, and macrophages. The cells were then plated in 6-well plates ($0.2x10^6$ cells/ml 4 ml/plate) in a complete medium (RPMI 1640, 10% heat-

inactivated FBS, 2mM L-glutamine, 10mM Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate) with addition of 1000U/ml mGM-CSF and mIL-4 (ENDOGEN, Woburn, MA). At Day 3, an additional dose of cytokines was added to the cell cultures. For adenoviral infection, DC were collected on day 5 and washed in serum-free medium. Virus was added directly to the pellet (10^9 pfu/10^6 DC) and the cells incubated for 1 h at 37°C before plating in a complete medium.

Flow cytometry. For phenotypic analysis of bone marrow-derived DC (Day 7), FITC- or PE-conjugated monoclonal antibodies recognizing murine CD11c, CD80, CD86, MHC class I and class II, and CD40 molecules were used. After incubation with antibodies for 30 min at 4°C, cells were washed with Phosphate Buffer Saline (PBS) and analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson, San Diego, CA). As a control to determine the percentage of transduced DC, cells were infected with Ad-EGFP 48 hours before FACS analysis.

IL-12 detection. IL-12 production by DC was determined by p70 IL-12 ELISA. A standard curve was generated using recombinant murine IL-12 (R & D Systems, Minneapolis, MN). To induce IL-12 expression, DC cultures were treated with inactivated *Staphylococcus aureus* (0.1% v/v of essentially non-viable cell suspension), (Sigma, St Louis, MO) 20 µl/well per $1x10^6$ DC cells.

Cytotoxic T lymphocyte assay. To evaluate the levels of CTL activity, splenocytes were pooled from control mice or mice treated with control DC or DC transduced with Ad-LacZ or Ad-CD40L, 7 days post intratumoral injection of DC. These spleen cells were re-stimulated *in vitro* with the irradiated (6000 rad) TS/A cells at a responder to stimulator ratio of 10:1 in culture medium supplemented with 10 IU/mL IL-2. Cytolytic activity was assayed after 6 days of

incubation. Target cells (TS/A, YAC-1 cells) were labeled with 100uCi/1x10^6 cells of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 1 h and plated in round-bottom 96-well plates at 1x10^4 cells/well. Effector cells were added at various E:T ratios in triplicate. The total reaction volume was kept constant at 200 µl/well. After cells were incubated for 4 h at 37 °C/ 5% CO_2, the ^{51}Cr release was measured in a gamma-counter. The amount of ^{51}Cr spontaneously released was obtained by incubating target cells in a medium alone. The maximum amount of ^{51}Cr incorporated was determined by adding 10% SDS. The percentage lysis was calculated as follows: % lysis = {(experimental release – spontaneous release) / (maximum release – spontaneous release)} x 100.

Data Analysis. Statistical analysis of experimental data was performed with a software package SigmaStat (STSS). For all analysis, the level of significance was set at a probability of 0.05 with results that had a p value less then the 0.05 considered significant. ANOVA was used to compare tumor growth for multiple groups of mice. All experiments were conducted with 5-7 mice per group receiving identical treatments that were repeated at least twice.

Results

Intratumoral injection of Ad-CD40L inhibited tumor growth in vivo. To examine the anti-tumor efficacy of local gene transfer of CD40L, a E1 and E3 adenoviral vector expressing murine CD40L was constructed as described in the Materials and Methods. To confirm expression of CD40L, MC38 tumor cells were infected with Ad-CD40L or the control vector Ad-ψ5. The infected cells were stained with anti-mCD40L antibody and analyzed by FACS analysis. Control MC38 tumor cells infected with Ad-ψ5 have no detectable surface expression of CD40L. However, cells infected with Ad-CD40L at an MOI of 100 expressed a high level of CD40L with more than 65% of cells positive for surface CD40L (Figure 1). The level of

transduction of MC38 with Ad-CD40L was similar to the level of the transduction efficiency observed with the control Ad-eGFP vector. Similar expression of CD40L (70%) was observed when TS/A tumor cells were infected with Ad-CD40L (data not should).

To determine whether intratumoral delivery of CD40L gene was able to confer an antitumor effect *in vivo*, a murine MC38 adenocarcinoma tumor model was utilized. Syngeneic C57BL/6 mice were inoculated subcutaneously with wild type MC38 tumor cells. On day 7, when the tumor size was 25 - 30 mm^2, 1 X 10^9 pfu of either Ad-CD40L or the control virus Ad-ψ5 were injected directly into the tumor. As shown in Figure 2A, non-treated and Ad-ψ5 treated mice had similar rates of tumor growth with average tumor sizes of 214 ±29 mm^2 and 228 ± 25 mm^2 respectively at day 27. In contrast, a single intratumoral injection of Ad-CD40L significantly inhibited tumor growth (83 ± 13 mm^2, $p<0.001$). These data demonstrate that a single intratumoral administration of adenovirus encoding mCD40L results in a marked suppression of tumor growth. Interestingly, the results of multiple administrations of Ad-CD40L, every 3 days for a total of three injections showed even more significant inhibition of tumor growth compare to results of a single injection of Ad-CD40L (Figure 2B).

To determine whether administration of Ad-CD40L into the tumor site might inhibit growth of a distant tumor through the induction of a systemic immune response, mice were inoculated with MC38 tumor cells into both right and left flanks. On day 7, the tumor on the left side was treated by intratumoral injection of either Ad-CD40L or a control virus. As shown in Figure 2C, only the Ad-CD40L-treated tumors displayed a significant delay in tumor growth (45 ± 7 mm^2) compared to control (191 ± 12 mm^2) and Ad-ψ5 (102 ± 10 mm^2) treated animals ($p<0.001$). In contrast, tumors on the left side showed no significant effect of CD40L-based therapy on the tumor growth (117 ± 11 mm^2). Taken together, these data suggest that the

treatment of MC38 tumor-bearing mice with an intratumoral administration of adenovirus encoding the mCD40L gene caused a significant inhibition of tumor growth *in vivo*. However, the antitumor effect was associated with local, but not systemic effects.

To evaluate whether the therapeutic effect of direct, intratumoral injection of Ad-CD40L was tumor specific, a Balb/c syngeneic breast tumor cell line TS/A was used. The TS/A cells were inoculated on Day 0 and palpable tumors injected with Ad-CD40L on Day 7. As shown in Figure 2D, growth of the TS/A tumor was significantly inhibited by the treatment with Ad-CD40L (78 ± 9 mm^2) compared to either the untreated control group (201 ± 13 mm^2) or to the group treated with control Ad-LacZ (152 ± 17 mm^2) ($p<0.005$). These results demonstrate that the antitumor effect induced by Ad-CD40L is not MC38 tumor or C57BL/6 strain specific. However, the antitumor effect of Ad-CD40L treatment was less effective in the TS/A model than the MC38 model.

Transduction of DC with Ad-CD40L in vitro. We have recently demonstrated that tumor cells secrete soluble factors that are able to reduce expression of CD40 on DC and consequently, blocking DC maturation (22). Moreover, our results suggest that DC obtained from tumor bearing mice express lower levels of co-stimulatory molecules resulting in reduced ability to stimulate T cell activities. Since it has been recently shown that CD40 ligation on DC results in increased expression of CD40 and enhanced DC function, it is possible that overexpression of CD40L on DC may be beneficial for function of CD40-deficient tumor-derived DC by an autocrine/paracrine up-regulation of their maturation in the tumor microenvironment. To test the efficacy of DC transduction, murine bone marrow-derived DC were transduced with Ad-CD40L on day 5 and analyzed by flow cytometry on day 7 (Figure 3A). Transduction of bone marrow-derived DC with Ad-CD40L resulted in expression of

CD40L in greater than 85% of the DC. Similarly, greater than 90% of the DC were eGFP positive following infection with Ad-eGFP (Figure 3A) and almost 89% of the DC generated from Balb/c mice were CD40L positive after Ad-CD40L transduction (data not shown).

To examine the effect of CD40L gene transfer to DC, the morphology and phenotype of the transduced cells were analyzed. DC derived from control bone marrow cultures exhibited the veiled dendrite morphology typical for DC and displayed a characteristic set of DC surface markers (Figure 3B). The genetically modified control DC expressed similar levels of the MHC class I and II molecules, the co-stimulatory molecules CD80, CD86, ICAM-1 adhesion molecules, and integrin CD11c. Infection of DC with Ad-CD40L at a 100 MOI resulted in a marked increase in levels of expression of CD80, CD86, CD40 and MHC class I molecules. For example, 53% of CD40L-transduced DC expressed CD86 molecules in comparison with 20% and 26.9% in non-transduced and Ad-ψ5 cells, respectively. Similar results were observed for CD80 expression. Interestingly, expression of MHC class II molecules on DC was also increased upon CD40L transduction confirming that overexpression of CD40L on DC stimulates their maturation by an autocrine and/or paracrine manner.

The interaction between CD40/CD40L is an essential trigger for IL-12 production by DC, important for induction of Th1 responses *in vivo* (23) and regulation of Th1/Th2 balance (24). To evaluate the level of IL-12 production by the different populations of genetically modified DC, cells were treated with saline or infected with Ad-LacZ or Ad-CD40L. DC infected with Ad-IL-12 were used as a positive control. 24 h post-transduction, both control and transduced DC cultures were treated with heat-inactivated *Staphylococcus aureus* (*S. aureus),* a potent inductor of IL-12 expression in cultured DC (21). The results revealed only a slight difference in *S.aureus*-induced IL-12 production between control DC and DC transduced with Ad-LacZ.

However, DC infected with Ad-CD40L produced a significantly higher level (5500 ± 427 pg/ml) of IL-12 ($p<0.01$) that was similar to the level of IL-12 production by Ad-IL-12-infected DC (5400 ± 568 pg/ml) (Figure 3C). The level of IL-12 production from the Ad-CD40L and Ad-IL-12 infected DC could be further simulated with treatment with *S.aureus.*

To demonstrate that overexpression of CD40L on transduced DC caused an activation of DC by an autocrine/paracrine manner, we infected bone marrow-derived DC with Ad-CD40L vector and evaluated the cluster formation in control and treated cultures. Non-infected DC and DC transduced with Ad-ψ5 vector served as controls. As shown on Figure 3D, CD40L-expressing DC formed multiple spontaneous clusters (panel C), the numbers of which were significantly higher than in control groups ($p<0.01$). In contrast, DC from control groups did not organized themselves in such structures. Therefore, these results demonstrate that transduction of DC with Ad-CD40L induces clustering of these cells and support our hypothesis that CD40L-transfected DC might be activated by autocrine or paracrine manner. Taken together, our data demonstrate that it is likely that CD40L-transfected DC activated each other in the tight clusters by multiple CD40 ligations resulting in accelerated maturation of cells displayed as an increase in expression of the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2), MHC molecules and increased production of IL-12.

Transduction of DC with Ad-CD40L increases their antitumor activity in vivo. Our *in vitro* results suggested that overexpression of CD40L on DC may be beneficial for the induction of antitumor immunity since it should induce DC maturation and function. To examine the antitumor efficacy of CD40L overexpressing DC, bone marrow-derived DC were infected with Ad-CD40L and injected directly into Day 7 MC38 tumors. As controls, tumors were treated with either DC transduced with Ad-ψ5 vector or with untreated non-transduced DC. As expected,

mice in both control groups developed rapidly growing tumors (untreated DC: 401 ± 21 mm^2; Ad-LacZ: 302 ± 18 mm^2). In contrast, mice immunized with DC transduced with Ad-CD40L, showed a significant inhibition (162 ± 17 mm^2) of tumor growth ($p<0.001$) (Figure 4A). These data demonstrate that a single intratumoral administration of DC/CD40L induces a strong antitumor response *in vivo*.

To evaluate the antitumor effect of DC/CD40L in a different tumor model and another strain of mice, Balb/c mice were inoculated with TS/A breast carcinoma cells and DC/CD40L were administered intratumoraly. Since we have previously demonstrated that Ad-IL-12 infected DC display a strong antitumor activity in a local therapy model (25, 26), treatment of TS/A tumor with Ad-IL-12-infected DC served as an additional positive control. As shown in figure 4B, animals in both control groups, and mice treated with control DC or DC infected with Ad-LacZ developed rapidly growing tumors (398 ± 23 mm^2 and 312 ± 28 mm^2, respectively) by Day 32. Animals in DC/IL-12 group showed a significant suppression (103 ± 15 mm^2) of tumor growth ($p<0.005$). However, mice treated with DC/CD40L displayed a complete tumor rejection with 5 out of 6 mice being tumor-free on day 23. These experiments were repeated twice with the similar results. Our data clearly demonstrate that DC genetically modified by Ad-CD40L infection confer a significant antitumor effect in the TS/A tumor model following intra-tumor injection. Moreover, the results suggest that DC/CD40L treatment is more effective than DC/IL-12 in conferring a sustained antitumor response.

To determine whether tumor rejection induced by DC/CD40L is accompanied by the induction of a specific immune memory, DC/CD40L-treated tumor-free mice were challenged with either TS/A tumor cells or a non-specific control tumor breast adenocarcinoma 4-T.1 cells on day 35. Additional controls included administration of both tumor cell lines into naive

syngeneic mice. No tumor growth was observed following TS/A tumor re-challenge only in mice previously treated with DC/CD40L (Figure 4C). All other groups of mice developed tumors at the expected growth rate. These results demonstrate that Balb/c mice treated with intratumoral DC/CD40L developed a specific, systemic antitumor immune response and immune memory.

To rule out the possibility that DC/CD40L might have a direct inhibitory effect on tumor cells, TS/A tumor cells ($5x10^5$ cells/well) were incubated in the presence of either unmodified DC or DC transduced with Ad-CD40L at different ratios (1:10 and 1:50 effector : target ratio) (data not shown). The growth rate of the tumor cells was analyzed using ^{3}H-thymidine incorporation. The results of these experiments showed no significant effect of all tested DC groups on tumor cell growth *in vitro*. Thus the significant antitumor effects observed following DC/CD40L treatment *in vivo* appear not to be mediated through a direct effect of DC on tumor cells.

Treatment with Ad-CD40L elicited cytotoxic T lymphocyte (CTL) response. It has been previously demonstrated that CD8+ T cells play a key role in the tumor regression after administration of an Ad-CD40L vector (27). Then we wanted to determine whether treatment with DC transduced with CD40L will be able to stimulate CTL response against tumor cells. Tumor-bearing Balb/c mice were inoculated with untreated DC or DC transduced with either Ad-LacZ or Ad-CD40L, intratumorally. Splenocytes were harvested 6 days later and analyzed for the specific CTL activity against TS/A or irrelevant Yac-1 cells. The results shown in Figure 6 demonstrate that there was no significant CTL activity against TS/A cells in animals treated with control or LacZ-transduced DC. In contrast, Ad-CD40L transduction significantly enhanced specific cytolytic activity (at 20:1 Effector : Target ratio: Control, 16 ± 6%; LacZ, 21 ± 3%; CD40L, 58 ± 3%). These cells however, did not exhibit cytotoxicity against Yac-1 cells. Taken

together, these results demonstrate that the vaccination of mice with DC transduced with Ad-CD40L can induce a long-lasting immune response with an activation of a CTL response directed against the nonimmunogenic cell line TS/A.

Discussion

The absence of effective conventional therapy for most cancer patients justifies the application of novel, experimental approaches. One alternative to conventional cytotoxic and hormonal agents is to promote the ability of the immune system specifically to target and eliminate tumor cells on the basis of expression of tumor-associated antigens (TAA). TAA could be presented to T cells by APC that generate a more efficient and effective antitumor immune response. In fact, it has been well documented that dendritic cells are capable of recognizing, processing and presenting TAA, in turn initiating a specific antitumor immune response (28-30). DC have been shown to activate tumor specific T cells both *in vitro* and *in vivo*, inducing both protective and therapeutic immunity, stimulating tumor rejection in several animal tumor models. However, results from several laboratories and clinical trials (15, 31, 32) suggested a significant, but still limited efficacy of TAA-pulsed DC administered to tumor-bearing hosts. This raises the question of how to improve or develop new DC-based immunotherapies for cancer. For instance, intratumoral administration of DC has been recently suggested as a novel experimental approach to treat cancer and demonstrated therapeutic efficacy in a first clinical trial (33). Combination of intratumoral injection of DC with cytokines might further increase the efficacy of DC-based therapies. For example, an intratumoral injection of DC in combination with the low dose of adenoviral vector encoding TNF-α elicited marked tumor suppression without toxicity and tumor-specific immune responses in four tumor models (34). Using adenoviral vector of CD40L and the number of DC that alone had no effect on tumor growth,

Kikuchi et al. have reported that the growth of CT26 colon adenocarcinoma and B16 melanoma murine s.c. tumors is significantly suppressed by direct administration of DC into established tumors that had been pretreated with Ad-CD40L two days previously (35).

The ability of CD40L gene transfer to stimulate antitumor immunity *in vivo* has been demonstrated previously by several groups. Stable expression of CD40L on MC38 colon carcinoma cells and MCA 205 fibrosarcomas resulted in inhibition of tumor growth following inoculation of the genetically modified tumor cells (17, 18). Similarly, it has been demonstrated that the *in vivo* genetic modification of tumor cells to express CD40L will trigger CD40 on local antigen-presenting cells to present tumor antigen to T cells, thus eliciting antitumor immunity to suppress growth of the tumor (36).

In this report, we have examined the antitumor effects of two different methods of intratumoral delivery of the CD40L gene, either directly by adenoviral injection or indirectly by injection of genetically modified DC. Our results demonstrate that the treatment of both MC38 and TS/A tumor-bearing mice with an intratumoral administration of adenovirus encoding the mCD40L gene caused a significant inhibition of tumor growth. However, this approach induced only a local, not systemic, antitumor effect, suggesting that Ad-CD40L did not elicit specific immune response. Similarly, it has been shown that Ad-CD40L treatment of established AC29 tumor induces a significant antitumor effect (37) and intratumoral injection of one of two synchronous tumors resulted in regression of both. However, Yanagi et al. demonstrated that Ad-CD40L treatment of hepatocellular carcinoma induces only a weak antitumor immunity (38). These data is not in agreement with our results since we have shown that Ad-CD40L considerably suppresses tumor growth.

Furthermore, we have demonstrated that the use of Ad-CD40L infected DC resulted in a significant anti-tumor response in MC38 tumor model, cause a complete rejection of TS/A tumors in 80% of mice and induced a specific systemic immunity. These data were similar to Kikuchi's, who showed that DC genetically modified to express CD40L elicit strong antitumor effect in B16 melanoma tumor model (39). In addition, it has been demonstrated that simultaneous administration of Ad-CD40L and naïve DC induces tumor regression (35).

CD40L is known to activate DC and stimulate their maturation, survival and secretion of IL-12 (3, 40). Therefore, DC transduced with Ad-CD40L appear to undergo self-activation, either through an autocrine or paracrine pathway, enhancing their function following injection into the tumor mass. In addition, it is likely that Ad-CD40L infection increased the survival and maturation of DC within the tumor microenvironment. In fact, it has been reported that CD40L expression on tumor cells is able to up-regulate Bcl-xl on DC, extending their lifespan.

Our data also demonstrate that expression of CD40L after infection reproducibly increased levels of CD80, CD86 and class II expression on DC, which is in agreement with multiple studies (40-42). This was an important observation, because co-stimulatory molecules play a major role in T cell activation by DC. We, also have shown that DC modified to overexpress CD40L produce high amounts of IL-12, which is plays a critical role in regulation of Th1/Th2 balance and in generation of antitumor immunity by DC.

In addition, the adhesiveness of DC was altered by CD40L expression with the modified cells becoming organized into clusters. Therefore, it is likely that CD40L-transfected DC activate each other in tight clusters by multiple CD40-CD40L interactions, resulting in accelerated maturation of cells reflected by the increased expression of co-stimulatory molecules CD80, CD86, and MHC molecules, as well as increased production of IL-12. There results are

consistent with previous reports where ligation of CD40 on DC by recombinant CD40L triggered the production of extremely high levels of bioactive IL-12 (40) and up-regulated the expression of ICAM-1, CD80, and CD86. These effects of CD40 ligation resulted in an increased capacity of DC to trigger proliferative responses and IFN-gamma production by T cells.

We previously have demonstrated that tumor cells significantly inhibit CD40-mediated signaling in DC, which, in turn, results in low expression of co-stimulatory and MHC molecules, low production of IL-12, and inability to induce an efficient proliferation of T cells (22). In this study, we have shown that overexpression of CD40L on DC is able to stimulate the ability of DC to induce antitumor immunity in several murine tumor models. However, the mechanisms of induction of antitumor immunity of genetically engineered DC during intratumoral vaccination remain unclear. It is also unclear if CD40L transfer to DC from tumor-bearing mice is able to stimulate a significant antitumor response. Experiments to determine if gene transfer of CD40L to DC isolated from tumor bearing mice with reduced CD40 expression results in an activated DC phenotype and induction of antitumor immunity following intratumoral injection are currently underway.

We have demonstrated that CD40L-transduced DC induce higher levels of cytotoxic T cell activation *in vivo* when compared to control cells, demonstrated by standard ^{51}Cr-releasing assay. These results in agreement with others which demonstrated that transduction of DC with Ad-CD40L stimulates cytotoxic T lymphocytes (43). Therefore, these data serve as an additional support for our main findings demonstrating that DC modified with CD40L gene elicit a strong antitumor effect *in vivo.*

Given our previous observation that DC genetically modified to express CD40L or IL-12 are able to induce a strong anti-tumor response following intratumor injection, we have been

comparing different immunostimulatory molecules for their ability to stimulate DC function in vivo. Initial analysis examining other members of the TNF family including RANKL and 4-1BBL suggest that CD40L gene transfer is more effective in inducing an antitumor response in MC38 and TS/A models. Moreover, CD40L gene transfer to DC appears to be more effective than DC gene transfer of IL-12, IL-18, GM-CSF or B7.1 for induction of a significant antitumor response following intratumor injection (16).

Overall, the data in this study provide supporting evidence for the concept of antitumor immunotherapy based on the administration of Ad-CD40L or DC transduced with Ad vector encoding CD40L. Immunotherapy with modified DC playing a key role in the induction of specific antitumor immune responses seems to be one of the promising alternative approaches in the treatment of cancer. Genetically modified DC overexpressing CD40L protein may serve as a basis for all DC-based clinical protocols, including the development of therapies for patients with cancer, HIV and infectious diseases.

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PERSPECTIVE

Sleuthful Pharmacology

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How many times have you found yourself in the position of not knowing how your favorite novel compound really works in a cell or organism? Your hypothesis, so carefully constructed, just does not seem to align with the data. You dream wearily of the day when a useful, if not universal, approach for determining the real mechanism of action becomes available. The current article, authored by T. Efferth and coworkers from nine international laboratories (Efferth et al., 2003), provides a clever illustration of one such approach. Armed with a semisynthetic derivative of artemisinin, the active principle of the *Artemisia annua* (sweet wormwood), the authors exploit a valuable public asset developed by the Developmental Therapeutics Program of the U.S. National Cancer Institute to decipher the mechanism of action of artesunate (Fig. 1), an antimalarial agent with previously described anticancer activity (Efferth et al., 2001). Artesunate is not a new agent; it is first-line therapy for *Plasmodium falciparum* and *Plasmodium vivax* malaria in some areas of Asia and also displays antischistosomal properties. More than 200 peer-reviewed articles on artesunate have been published in the last seven years, a testimony to its importance as a therapeutic agent. Nonetheless, only three of those references refer to its potential use as an anticancer agent.

The work of Efferth et al. (2003) illustrates how a hypothesis-generating (derisively termed "ignorance-based" by some) approach rather than a hypothesis-driven approach can yield useful insights into the possible mechanisms of action of a new anticancer compound. The National Cancer Institute has tested tens of thousands of compounds against their 60-tumor cell panel for growth inhibition and has provided additional information about the molecular phenotype of each cell line. Information from this substantial undertaking has been placed in the public domain on a readily accessible website (http://dtp.nci.nih.gov) that can be mined with the National Cancer Institute's COMPARE program. As Efferth et al. (2003) showed, this informatics tool can generate testable candidate molecular targets for a compound that does not have any obvious relationship, at least based on this algorithm, to clinically used anticancer drugs. Rapidly growing tumors were more sensitive to artesunate than slowly growing tumors, but this is seen with many existing anticancer agents with the exception of the drugs that cause DNA adducts, such as carboplatin, dacarbazine, and isosfamide.

With artesunate, the authors selected 465 genes whose expression levels were obtained by microarray hybridization and are available in the National Cancer Institute's data base. They used hierarchical cluster analysis and found 60 genes whose expression correlated with sensitivity or resistance to artesunate. Three genes were studied in greater detail because of the high correlation of their cDNA levels with a cytotoxic response to artesunate, their mathematically determined low false positive discovery rate, and the availability of appropriate cell systems to test their importance. All three selected gene products were validated as genes involved in the artesunate cytotoxic response using gene transfer methodology. Transfection of cells with the cDNA for epidermal growth factor receptor, the target of the recently approved anticancer agent gefitinib (Iressa), and γ-glutamylcysteine synthetase altered sensitivity to artesunate. The authors also used a tetracycline repressor expression vector system, first developed by Blomberg and Hoffmann (1999), to confirm a role for the CDC25A gene in the cytotoxic mechanism of artesunate. This latter observation is particularly interesting because, like the epidermal growth factor receptor, the Cdc25A protein has been shown to be over-expressed in a number of human tumors, including breast cancer (Cangi et al., 2000), and has been implicated in several aspects of the malignant phenotype (Fig. 2). Thus, Cdc25A controls cell cycle checkpoints that regulate progression through G_1/S, S, and mitosis due to its ability to dephosphorylate and, thus, activate cyclin-dependent kinases. Consequently, elevated levels of functional Cdc25A are thought to allow cells to replicate and duplicate damaged DNA and, thus, to encourage genetic instability. Cdc25A also has been shown to block apoptosis signal-regulating kinase-1 (ASK-1) (Zou et al., 2001) and to affect epidermal growth factor receptor (Wang et al., 2002), raf-1 (Xia et al., 1999), and steroid

ABBREVIATIONS: ASK-1, apoptosis signal-regulating kinase-1.

receptors (Ma et al., 2001). The interaction between Cdc25A and ASK-1 or steroid receptors did not seem to require a Cdc25A protein with phosphatase activity. Thus, an agent that preferentially affects cells that over-express Cdc25A is of considerable pharmacological interest (Lyon et al., 2002). Efferth et al. (2003) also show that the growth-inhibitory activity of artesunate was not influenced by the most common cellular multidrug resistance mechanisms or by the p53 or p21 status of cells.

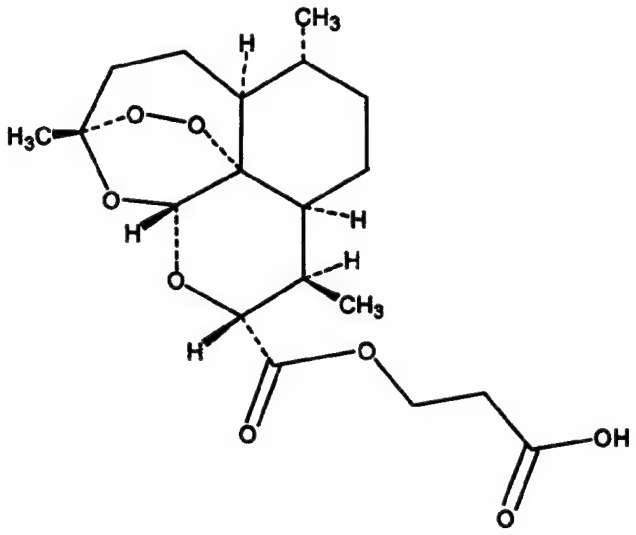

Fig. 1. Chemical structure of artesunate.

As with any manuscript, the current contribution is not without potential issues. For example, the fundamental tool for selecting the candidate genes was cDNA microarray data. No persuasive evidence was provided that glutamate-cysteine ligase regulatory subunit, epidermal growth factor receptor, and CDC25A protein expression was elevated in concert with the cDNA levels. Moreover, if one accepts that the Cdc25A acts as an oncogene because it promotes genetic instability, then it is difficult to deduce theoretically how the very transient Cdc25A over-expression occurring after tetracycline withdrawal could render a cell more sensitive to artesunate. Perhaps artesunate was acting to disrupt Cdc25A interactions with ASK-1, raf-1, or epidermal growth factor (Fig. 2). The current article provides no mechanistic information on how the candidate gene products alter sensitivity to artesunate. Furthermore, the failure to see any effect of Cdc25A expression on doxorubicin-induced growth inhibition is not fully in agreement with a recent report (Xiao et al., 2003), which was published after the manuscript by Efferth et al. was submitted. Xiao et al. (2003) demonstrated that

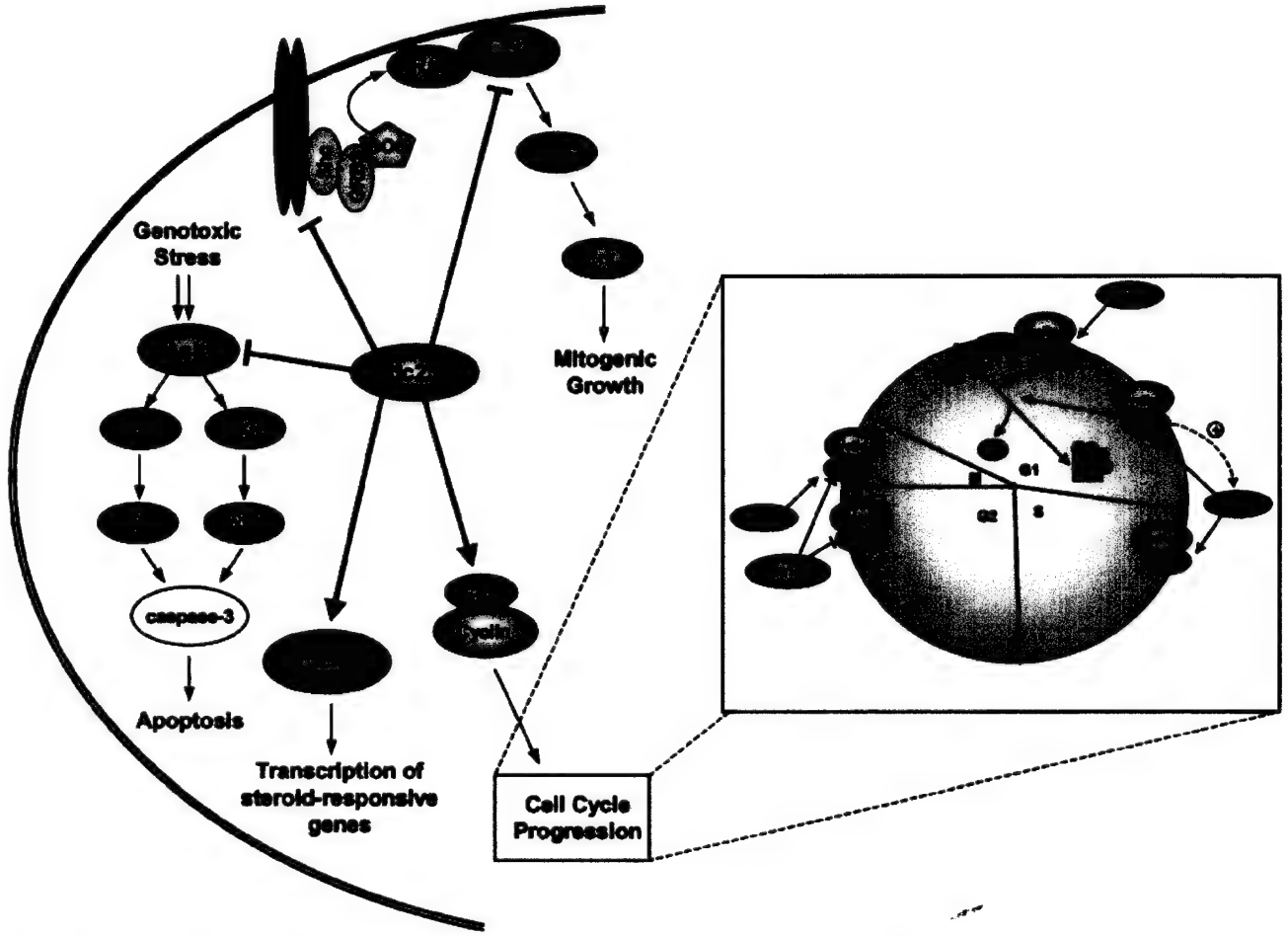

Fig. 2. Potential intracellular actions of Cdc25A. Evidence for potential positive (arrows) and negative (T-bar) effects of Cdc25A are indicated. The role of Cdc25A on cell cycle progression is indicated in the right inset.

high expression of Cdc25A caused resistance to doxorubicin, whereas low-level expression did not alter doxorubicin sensitivity. Although the difference in results of the two groups might reflect the use of different species, knowledge about the Cdc25A protein expression levels in the Efferth study would be useful comparative information.

Like many good articles, the hypothesis-generating study of Efferth et al. provides as many new questions as it answers. What was the mechanism of action by which γ-glutamylcysteine synthetase, epidermal growth factor receptor, and Cdc25A phosphatase affected cell sensitivity to artesunate? What about the other 57 genes? How does one determine the hierarchical importance of the 60 identified genes, really, in the ultimate antiproliferative activity of artesunate? These and other questions may arise in the mind of the readers of this article, perhaps stimulating them to answer these questions or even emulate the sophisticated approach taken by the authors of this contribution.

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DUAL-SPECIFICITY PHOSPHATASES AS TARGETS FOR ANTINEOPLASTIC AGENTS

Michael A. Lyon, Alexander P. Ducruet‡, Peter Wipf* and John S. Lazo‡*

Dual-specificity protein phosphatases are a subclass of protein tyrosine phosphatases that are uniquely able to hydrolyse the phosphate ester bond on both a tyrosine and a threonine or serine residue on the same protein. Dual-specificity phosphatases have a central role in the complex regulation of signalling pathways that are involved in cell stress responses, proliferation and death. Although this enzyme family is increasingly the target of drug discovery efforts in pharmaceutical companies, a summary of the salient developments in the biology and medicinal chemistry of dual-specificity phosphatases has been lacking. We hope that this comprehensive overview will stimulate further progress in the development of small-molecule inhibitors that could form the basis for a new class of target-directed therapeutic agents.

The regulation of cellular signalling pathways by kinases and phosphatases is an extremely active current field of research in academia. Small-molecule inhibitors, many based on natural products, are becoming available that allow the reversible and graded regulation of these enzyme families. Such compounds are valuable tools to probe the function of kinases and phosphatases in both diseased and normal tissues and cells. In the pharmaceutical industry, recent efforts to inhibit the breakpoint cluster region (*BCR*)–Abelson leukaemia viral oncogene (*ABL*) gene mutation product have culminated in the discovery of the therapeutic effects of Gleevec (Glivec, STI-571) and have therefore validated the kinase family as a high-quality clinical target for drug development[1]. Although such a proof-of-principle remains to be established for the phosphatase family, the clinical success of Gleevec has invigorated drug discovery efforts in this field as well.

Eukaryotic cellular processes, such as transcriptional regulation, apoptosis, protein degradation, nuclear transport and cell-cycle control, are crucially dependent on signal-transduction pathways[2,3]. A central mechanism by which mammalian cells relay signals is through the formation and hydrolysis of phosphate esters on tyrosine, serine and threonine residues in signal-transduction proteins. The phosphorylation status of proteins is maintained by protein kinases, which catalyse the formation of phosphate ester bonds, and by protein phosphatases, which catalyse the hydrolysis of phosphate ester bonds[3,4]. Initially, protein kinases dominated the signal-transduction 'limelight', and protein phosphatases were relegated to a 'house-keeping' function to counteract protein-kinase activity. We now recognize that kinases and phosphatases have equally important roles in phosphorylation-mediated signal transduction, and that protein phosphatases are regulated in a highly sophisticated manner[2,3,5].

The eukaryotic protein-phosphatase family has been classified into two main groups according to substrate preference. The serine/threonine-specific protein phosphatases (PS/TPases) are metalloenzymes that hydrolyse phosphate ester-modified serine or threonine residues[4,6]. PS/TPases comprise several subunits: a catalytic subunit that has a metal ion at its centre and one or more regulatory subunits[6]. The proposed mechanism of phosphate ester hydrolysis involves attack of the phosphorus atom by a metal-activated water molecule, and proceeds without the formation of a phosphoenzyme intermediate[6,7]. Enzymes that specifically hydrolyse phosphate ester-modified tyrosine residues

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make up the second group of the protein-phosphatase family, collectively known as the protein tyrosine phosphatases (PTPases). As a group, PTPases have a vital role in intracellular signal-transduction pathways and regulate such physiological processes as cell growth and proliferation, cell-cycle progression, cytoskeletal integrity, differentiation and metabolism[3,4]. PTPases are typified by the presence of an absolutely conserved active-site motif — His-Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Arg — that falls within the catalytic domain of the enzyme. Outside their catalytic domains, the PTPases contain little amino-acid homology, which presumably reflects their diverse roles and multiple substrates[6]. Unlike PS/TPases, PTPase phosphate ester hydrolysis is metal-ion independent. In a two-step process, PTPases catalyse the formation of a transient phosphoenzyme intermediate by transferring the phosphate to a catalytic cysteine residue and then expelling the dephosphorylated substrate from the active site using an acidic amino-acid residue to protonate a tyrosine phenolic oxygen[6]. The active PTPase is regenerated when an amino acid functioning as a general base activates a proximal water molecule, allowing hydrolysis of the phosphoenzyme intermediate and resulting in the release of inorganic phosphate[6,8].

Dual-specificity phosphatases (DSPases) are a PTPase subclass that are uniquely able to hydrolyse the phosphate ester bond on both a tyrosine residue and either a serine or threonine residue located in the same protein. The active site of the prototypical DSPase DSP3 (also known as VHR; vaccinia virus phosphatase VH1-related protein) is a shallow cleft that is ~6 Å in depth, compared with the deeper ~9 Å cleft found in PTPases, which presumably allows the DSPase to accommodate less extended phosphoserine and phosphothreonine substrates[6,9,10]. DSPases have crucial roles in intracellular signal-transduction pathways and are most prominently known for regulating the mitogen-activated protein kinase (MAPK) signalling pathways and cell-cycle progression. The prototypic DSPase VHR was recently found to function as a constitutive nuclear MAPK phosphatase[11]. Whereas the MAPK phosphatases typically function in negative-feedback loops to downregulate MAPKs after their activation, VHR might function in quiescent cells to prevent the untimely activation of the extracellular-signal regulated kinase (ERK) MAPKs and inactivate them once they enter the nucleus[11].

The cell-division cycle 25 (CDC25) family of DSPases regulates cell-cycle progression by dephosphorylating and activating cyclin-dependent kinases (CDKs). Inactive CDKs are phosphorylated at adjacent threonine and tyrosine residues near their amino termini, and dephosphorylation at both sites by CDC25 phosphatases catalyses their activation and allows the CDKs to propagate cell-cycle signal transduction[12–14]. Because of the unique biochemical and regulatory properties of CDC25, the remainder of this review will focus on the biology of the CDC25 phosphatases, their involvement in neoplastic transformation and current efforts to develop small-molecule inhibitors for potential therapeutic intervention.

wee1
A nuclear kinase that inactivates cyclin-dependent kinase 1.

Early identification

cdc25 was first identified in yeast as the twenty-fifth protein that influenced the cell-division cycle[15]. It was noted that on mutation of *cdc25*, cells assumed the opposite of a *wee1* phenotype; that is, they failed to divide and grew to an enlarged state. This indicated that *cdc25* functioned antagonistically to *wee1*, which was known to encode a kinase that promoted yeast cell division[15,16]. The three human homologues of *cdc25* (*CDC25A*, *CDC25B* and *CDC25C*; FIG. 1) were identified using degenerate-oligonucleotide-primer-based PCR cloning and by genetic complementation of a *cdc25*^{ts} temperature-sensitive yeast strain[17–19]. All three human homologues were successful in rescuing the temperature-sensitive yeast mutant, even though we now know that their expression is cell-cycle specific in mammalian cells: *CDC25C* messenger RNA is expressed predominantly in G2 and M phase, *CDC25B* mRNA is expressed throughout the cell cycle and is elevated in G2, and *CDC25A* mRNA is expressed mainly during late G1 and S phase[18–20].

Mammalian CDC25

The three human CDC25 isoforms, although sharing functional and sequence homology, are encoded by unique genes that localize to three different chromosomes: *CDC25A* is found on 3p21, *CDC25B* on 20p13 and *CDC25C* on 5q31. The human CDC25 family is further complicated by multiple splice variants: two for CDC25A, five for CDC25B and five for CDC25C[21,22]. The function of the CDC25 splice variants remains unclear, but it has been proposed that the splice variants of CDC25A and CDC25C might have different roles in different cell lines and differ in cell-cycle-phase distribution, and that the alternative splicing leads to the loss of consensus phosphorylation sites in the amino termini of the proteins[21]. Only two of the five CDC25B variants seem to be predominantly expressed in mammalian cells: CDC25B2 and CDC25B3 (REF. 22).

At the protein level, CDC25 phosphatases are structurally divided into two main domains. The carboxy-terminal domain comprises ~30% of the protein, is marked by a conserved Leu-Ile-Gly-Asp motif and is highly homologous between the three isoforms. The amino-terminal domain varies in length and contains very little homology between the three isoforms[23] (FIG. 1). The carboxy-terminal domain houses the catalytic site of the enzyme, which contains the canonical PTPase active-site motif His-Cys-Xaa$_5$-Arg (REF. 24). However, a recent debate has ensued over whether or not the CDC25 phosphatases use a general acid in their catalytic mechanism, as interestingly, an amino acid functioning as a general acid has not been convincingly identified and might even be located on the protein substrate[25,26]. The amino terminus contains phosphorylation sites, and might have a negative regulatory effect on CDC25 catalytic activity through an unknown mechanism; this negative regulatory effect is apparent in the increased phosphatase activity that is seen when the regulatory domain is removed from the CDC25A and CDC25B catalytic domains[25,27] (J. S. L. and A. P. D., unpublished observations).

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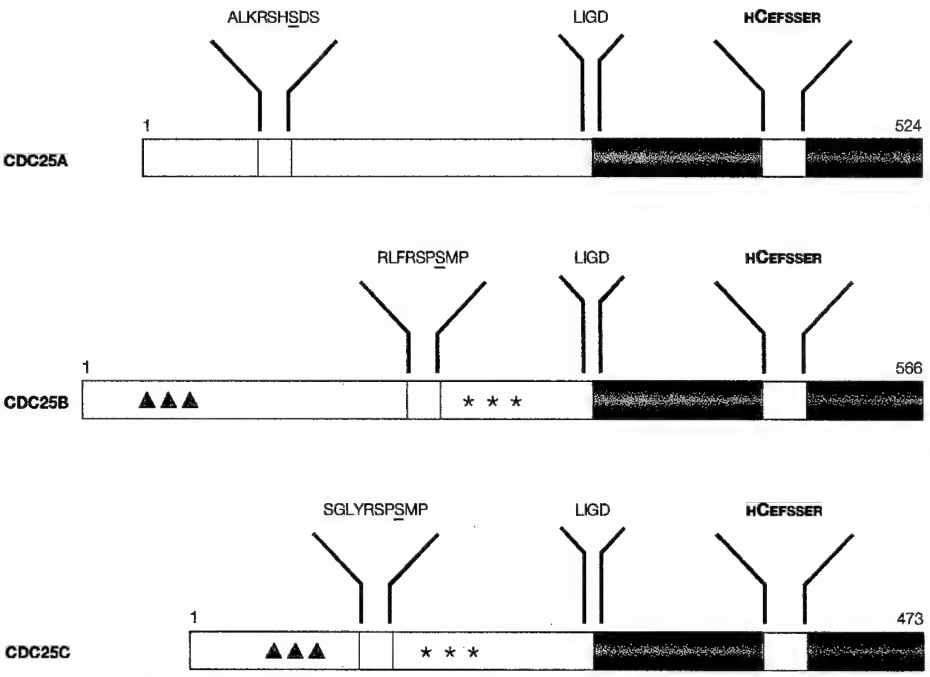

Figure 1 | **Mammalian CDC25 phosphatases.** The mammalian cell-division cycle 25A (CDC25A), CDC25B and CDC25C phosphatases are encoded by three separate genes and have non-redundant functions in the cell. They comprise the amino-terminal domain, which is the site of regulatory phosphorylations, and the carboxy-terminal domain, marked by a conserved Leu-Ile-Gly-Asp motif that contains the canonical protein tyrosine phosphatase (PTPase) active-site motif His-Cys-Xaa_5-Arg, for which Xaa is any amino acid. The catalytic activity of these enzymes might undergo negative regulation as a result of interactions between the amino- and carboxy-terminal domains. Single-letter amino-acid abbreviations are used, and underlined letters represent validated phosphorylation sites. The PTPase active-site motif is indicated by bold letters, with the catalytic cysteine residue in larger font. Identified nuclear-localization sequences are indicated with asterisks and known nuclear-export sequences are indicated with triangles.

The crystal structures of the catalytic domains of CDC25A and CDC25B have been reported at 2.3 Å and 1.9 Å resolution, respectively, but no crystal structure for the full-length protein is available. Both phosphatases contain the canonical His-Cys-Xaa_5-Arg PTPase catalytic-site motif nestled in the P-LOOP structural motif, a characteristic of all tyrosine phosphatases[8,26,28]. Although the overall structure of the catalytic domains of the two CDC25 phosphatases is similar, CDC25A failed to bind oxyanions in its catalytic site, whereas CDC25B readily bound tungstate and sulphate in its catalytic site in a mode similar to other PTPases and DSPases[28]. This might stem from the shallow nature of the CDC25A active site compared with the active-site architecture of CDC25B, which is more reminiscent of other DSPase active sites. The CDC25A catalytic domain also lacks any loops proximal to the active site that could facilitate substrate binding[26,28]. A comparison of the two crystal structures shows that the carboxy-terminal tail of CDC25B folds back on its active site, whereas the carboxy-terminal tail of CDC25A is directed away from the active-site cleft, which results in a more open structure[28]. These structural data lend credence to the recent biochemical data arguing that the final 17 carboxy-terminal residues of CDC25B confer its substrate specificity, and reports in the literature that propose a higher degree of promiscuity for CDC25A substrate selection[29–31]. Interestingly, although CDC25A has been functionally compared to other PTP- and DSPases because of its canonical His-Cys-Xaa_5-Arg motif in its active site, it unexpectedly has identical topology to the bacterial sulphur-transferase protein rhodanese. However, the significance of this homology is unclear[26]. By contrast, CDC25B compares more closely with other PTPases and DSPases[28]. Although the amino-acid sequences of the catalytic domains of CDC25A and CDC25B contain a high degree of homology, the differences in their active-site crystal structures indicate that designing inhibitors that are specific for the CDC25 isoforms should be possible.

P-LOOP
The phosphate-binding loop formed by the active-site motif of protein tyrosine phosphatases.

Biochemical regulation

Under normal physiological conditions, the phosphatase activity of CDC25 is thought to be subject to tight regulation. CDC25 phosphatases are known to undergo both stimulatory and inhibitory phosphorylation that influences their catalytic activity, subcellular localization and stability. Initial reports on the phosphorylation of CDC25A and CDC25C suggested a positive-feedback loop, in which the cyclin–CDK

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UBIQUITYLATION
A multi-step post-translational modification through which ubiquitin, a highly conserved ~76-amino-acid protein, is covalently linked to a lysine residue in a protein. Ubiquitylation targets proteins for degradation by the proteasome, in what is now called the classic ubiquitin-dependent proteolysis pathway.

CHECKPOINT
A point in the cell cycle at which intracellular conditions are self-inspected and the results determine whether progression through the cell cycle is allowed. There are two main cell-cycle checkpoints: G2/M and G1/S.

complex that was activated by a CDC25 would, in turn, phosphorylate and increase the activity of the CDC25 that activated it[32,33]. This seems to be a plausible hypothesis, but such a positive-feedback system is not used by all CDC25 isoforms[23]. CDC25B is also phosphorylated by one of the cyclin–CDK complexes it activates, cyclin A–CDK1, but this feedback phosphorylation event targets CDC25B for degradation by the proteasome[34,35]. Proteasome-mediated degradation also occurs when CHK1, a DNA-damage effector kinase, phosphorylates CDC25A and targets it for UBIQUITYLATION and proteasome-mediated degradation in response to genetic insults[36–39]. Phosphorylation has also been reported to influence the ability of the CDC25 phosphatases to interact with 14-3-3 proteins, which function to retain proteins in various subcellular compartments. Serine phosphorylation of the amino terminus of CDC25B by p38, and of CDC25C by CHK1 and CDC25C-associated protein kinase (C-TAK), generates 14-3-3 binding sites, which in turn seem to be responsible for sequestering CDC25 in the cytoplasm[40–46] (FIG. 2). Whether 14-3-3 proteins catalyse export from the nucleus or simply serve to sequester CDC25 in the cytoplasm remains unclear. Evidence to support the latter hypothesis exists in the form of nuclear-localization sequences (NLS) and nuclear-export sequences (NES) that are found in the amino termini of CDC25B and CDC25C, which alone are sufficient to catalyse transport into and out of the nucleus, respectively[40,42] (FIG. 1). This indicates that 14-3-3 proteins might retain CDC25 in the cytoplasm. Deletion of the NES in CDC25C increases CDC25C nuclear accumulation, but deletion of both the NES and the 14-3-3-binding site causes complete nuclear accumulation, implying a necessary role for 14-3-3 in the CDC25C nuclear-export process[42]. By contrast, deletion of either the NES or the 14-3-3-binding site in CDC25B causes a predominantly nuclear accumulation, indicating that 14-3-3 might have a more important role in the cytoplasmic accumulation of CDC25B[40]. It has been speculated that 14-3-3 proteins might also link CDC25A to the MAPK signalling cascade by facilitating interactions with the MAPK kinase kinase RAF1, an upstream activator of the MAPK cascade[30,47,48].

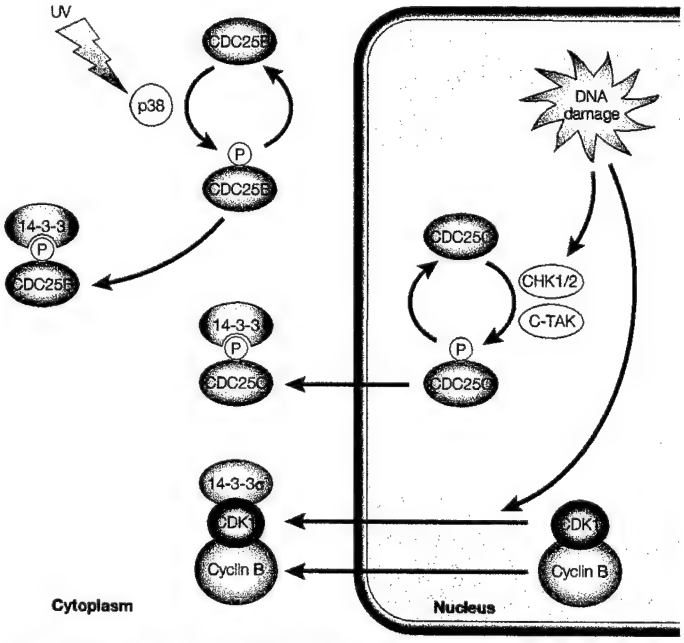

Figure 2 | **Spatial regulation of CDC25 phosphatases at the G2/M checkpoint.** In response to DNA damage or ultraviolet (UV) irradiation, cell-division cycle 25B (CDC25B) and CDC25C are serine phosphorylated, creating 14-3-3-binding sites; this leads to 14-3-3 binding and CDC25 cytoplasmic retention. 14-3-3σ sequesters cyclin B–cyclin-dependent kinase 1 (CDK1) in the cytoplasm. CDC25C-associated protein kinase (C-TAK)/β phosphorylates Ser216 of CDC25C independent of DNA damage. CDC25 cytoplasmic sequestration is independent of the tumour-suppressor protein p53, whereas 14-3-3σ-mediated sequestration of cyclin B–CDK1 is p53 dependent.

Regulation of the cell cycle

The cellular roles of the individual CDC25 isoforms in different phases of the cell cycle have been carefully analysed. The primary CDK substrate for CDC25A seems to be cyclin E–CDK2 (REFS 20,49). By activating this CDK, CDC25A has a primary function to promote transition through the G1/S cell-cycle CHECKPOINT and allow S-phase progression[20,49] (FIG. 3). Nonetheless, CDC25A protein levels and activity remain elevated past S phase, and might even increase as cells enter mitosis[39,49]. The functional significance of elevated CDC25A activity throughout G2 and mitosis is not clear, as most of the established CDC25A substrates are associated with G1 and the G1/S transition. Because the human CDC25C isoform is the most homologous to yeast and *Xenopus* Cdc25, most investigators believe that CDC25C functions primarily in mitosis and catalyses mitotic progression by activating cyclin B–CDK1 (REFS 12,14,50,51). As CDC25B can also activate cyclin B–CDK1, CDC25B was believed to be functionally redundant to CDC25C[52]. More recent work implicated CDC25B not only as an activator of cyclin B–CDK1 at the onset of mitosis, but also as an activator of cyclin A–CDK2 in late G2 (REFS 53,54). CDC25A and CDC25B might also have a role in terminally differentiated cells, as they have been observed to be overexpressed in the brains of patients with Alzheimer's disease, and have been implicated in neurodegeneration[55].

Cell-cycle checkpoints

Possibly the most crucial part of the cell cycle involves replicating (S phase) and separating (M phase) DNA, and cells have highly evolved mechanisms to halt the cell cycle to ensure high-fidelity DNA replication and distribution[56]. Not surprisingly, the CDC25 phosphatases are primary participants in these cell-cycle checkpoints. Increased Cdc2 (the equivalent to mammalian CDK1) Tyr15 phosphorylation in fission yeast first implicated CDC25 in the DNA-damage checkpoint[57]. Later, it became evident that checkpoint-mediated CDC25 inhibition was due to phosphorylation by CHK1 and CDS1/CHK2 — DNA-checkpoint kinases that are activated in response to DNA

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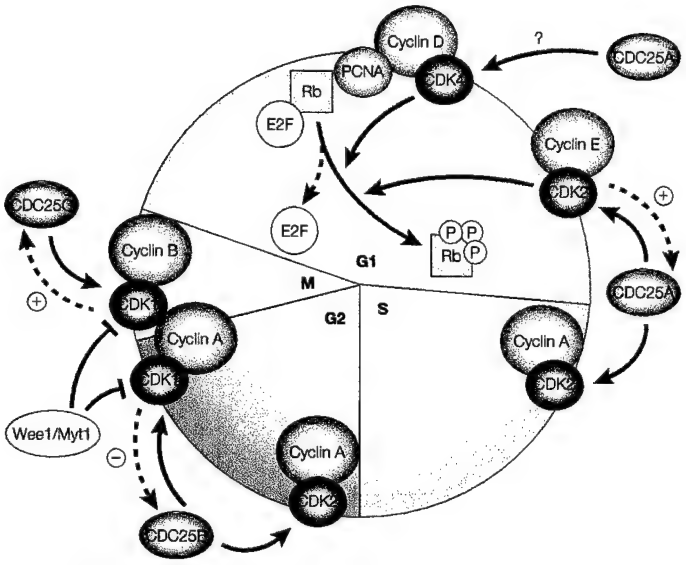

Figure 3 | **CDC25 phosphatases promote mammalian cell-cycle progression.** This simplified schematic representation of the cell cycle includes the main molecular participants that are involved in cell-division cycle 25 (CDC25)-mediated cell-cycle progression. Dotted arrows represent known feedback loops, either positive (+) or negative (–), as indicated. It is unclear whether cyclin D–cyclin-dependent kinase 4 (CDK4) is a bona fide substrate of CDC25A. E2F is a transcription factor that was originally identified through its role in transcriptional activation of the adenovirus E2 promoter. P, phosphate; PCNA, proliferating-cell nuclear antigen; Rb, retinoblastoma protein.

damage — and the generation of a 14-3-3-binding site, leading to cytoplasmic sequestration of CDC25 (REFS 44,45,58) (FIG. 2). This mechanism is conserved between yeast and mammals, with CDC25C being a primary target of the G2/M CHECKPOINT[59]. Certain specific genotoxic stresses induce a parallel pathway for cell-cycle arrest through inhibition of CDC25B; this occurs though phosphorylation of CDC25B by p38, a stress-responsive MAPK, and results in 14-3-3 binding and sequestration of CDC25B[60]. In addition, the primary mitotic cyclin–CDK, cyclin B–CDK1, is targeted by the G2/M checkpoint for cytoplasmic sequestration by associating with another 14-3-3 isoform, 14-3-3σ (REF. 61). A large component of the G2/M cell-cycle checkpoint involves 14-3-3-mediated cytoplasmic sequestration of the crucial cell-cycle regulatory components CDC25B, CDC25C and cyclin B–CDK1 (REFS 22,40,42,43,46). On the basis of the similarities of these responses to genotoxic stresses, it could be concluded that phosphorylation of CDC25 by a stress-responsive kinase and concomitant sequestration and inactivation by 14-3-3 proteins might be a general cell-cycle-arrest programme. However, genotoxic stress can also induce G1/S ARREST through CDC25A inhibition, which is independent of 14-3-3. In response to DNA damage from ultraviolet (UV) irradiation, ionizing radiation (IR) or interruption of DNA synthesis, CDC25A levels decrease rapidly by polyubiquitylation and proteasome-mediated degradation, resulting in G1/S cell-cycle arrest[36,37,39]. This checkpoint is a rapid response to genetic stresses, and is independent of the tumour-suppressor protein p53 (gene, *TP53*); the p53 checkpoint can be enacted as a second wave of cell-cycle arrest if necessary[37]. In response to UV and IR, CDC25A degradation is triggered by CHK1- or CHK2-mediated Ser123 phosphorylation; the serine phosphorylation site matches a consensus regulatory site that is highly conserved among CDC25 isoforms[36,37] (FIG. 1). Because of their unique roles in cell-cycle promotion, it is perhaps not surprising that cells have highly precise mechanisms that target the inactivation and destruction of CDC25 proteins to arrest the cell cycle and maintain genomic integrity.

G2/M CHECKPOINT
A point in the second gap phase (G2) of the eukaryotic cell cycle at which cell progression into and through mitosis (M phase) is determined.

G1/S ARREST
G1/S arrest occurs at the G1/S checkpoint, at which eukaryotic cells determine if progression from the first gap phase (G1) into and through the DNA synthetic phase (S phase) should occur.

p53
A potent tumour-suppressor protein of 53 kDa that participates in the G1/S checkpoint regulation.

YEAST-TWO-HYBRID SYSTEM
A method to identify the physical interaction between two proteins or peptides within yeast cells by detecting a change in transcriptional activity.

Role in malignancy

Deregulation of cell-cycle control proteins embodies several of the essential cellular alterations that typify malignant transformation[62]. Typically, accelerated cell-cycle progression can be promoted in tumour cells through one of two means, either loss of tumour-suppressor genes, such as the retinoblastoma gene (*Rb*) and *TP53*, the physiological function of which is to keep the cell cycle in check, or through amplification or mutation of proto-oncogenes, the main physiological function of which is to promote and relay growth signals to the nucleus. It should therefore not be surprising that CDC25 phosphatases, as vital promoters of cell-cycle progression, could function in promoting malignant transformation. Indeed, CDC25A and CDC25B have been reported to have oncogenic properties, transforming normal mouse embryonic fibroblasts in cooperation with an oncogenic isoform of Ras (Ha-Ras^{G12V}) or in an *Rb*$^{-/-}$ background[63]. Since this seminal report, overexpression of CDC25A and CDC25B has been documented in numerous human cancers, including breast, colon, gastric, head-and-neck and non-small-cell lung cancers, oesophageal squamous-cell carcinoma, colorectal carcinoma, non Hodgkin's lymphoma and neuroblastoma[63–71]. CDC25A overexpression is associated with poor survival in breast carcinomas[64].

There are several hypotheses to explain the oncogenic potential of CDC25A and CDC25B. Senescent human mammary epithelial cells arrest in G1 owing to down-regulation of CDC25A, which leads to cyclin E–CDK2 inhibition[49,72]. CDC25A overexpression in mammary epithelial cells might allow them to avoid senescent arrest and contribute to malignant progression. This hypothesis is supported by the observation that CDC25A antisense resulted in decreased CDK2 activity and inhibition of S-phase progression in MCF-7 breast-cancer cells[64]. CDC25A might also exert its oncogenic potential through its interactions with the proto-oncogene *RAF1* and by regulation of the MAPK signalling cascade[30,48,73]. CDC25A has been co-localized with RAF1 in the cytoplasm of cells, and the two proteins interacted in a YEAST-TWO-HYBRID SYSTEM; RAF1 phosphorylates and activates CDC25A *in vitro*, adding functional significance to their co-localization[47,48]. It was later

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Table 1 | **Mean inhibitory concentration (IC_{50}) of compounds against protein phosphatases***

Compound‡	IC_{50} values against various protein phosphatases				
	CDC25A (μM)	CDC25B (μM)	CDC25C (μM)	VHR (μM)	PTP1B (μM)
5	–	–	–	2.0	–
20 (*R*)	100	100	–	1.5	–
21 (*S*)	100	100	–	1.4	–
22 (*R*)	34	34	–	3.4	–
23 (*R*)	>100	>100	–	>100	–
24	–	0.38	–	4.0	–
25	–	0.52	–	4.6	–
26	–	0.60	–	4.7	–
27	–	0.40	–	12.4	–
28	2.8	–	–	4.6	4.4
29	4.2	–	–	10.8	11
30	8.4	–	–	7.9	17
31	3.8	–	–	4.6	5.4
35	–	–	5.1	–	–
36	–	–	16	–	–
37	–	–	0.8	–	–
38	–	–	1.5	–	–
39	–	–	6.8	–	–
40	–	–	2.4	–	–
41	–	–	6.1	–	–
42	–	–	9.0	–	–
44	2.2	–	–	–	–
45	2.4	–	–	–	–
46	2.5	–	–	–	–
47	1.9	–	–	–	–
48	2.9	–	–	–	–

*The inhibition of recombinant protein phosphatase was determined as described in REF. 123. ‡Compound numbers relate to the numbering in the main text. The mean inhibitory concentration (IC_{50}; μM) was obtained from 3–5 measurements ± standard error of mean (SEM). Values that lack ± are from a single determination. – indicates that the value has not been determined. CDC, cell-division cycle; PTP1B, protein tyrosine phosphatase 1B; VHR, vaccinia virus phosphatase VH1-related.

reported that CDC25A dephosphorylates RAF1 at tyrosine residues and inactivates RAF1 after its growth-factor-mediated activation[30]. These data indicate that activation of the RAS–RAF–MAPK cascade promotes activation of CDC25A, which subsequently downregulates RAF1 and terminates the initiating signal for MAPK activation, forming a negative-feedback loop and concomitantly priming the cell for cell-cycle progression by activating CDC25A. This might be functionally significant in cells transformed by *RAF1* or by an oncogene upstream of *RAF1*, because elevated constitutive activation of the MAPK cascade has been reported to have cytostatic and cytotoxic effects[73–76]. Therefore, CDC25A overexpression might not only accelerate cell-cycle progression but might also protect certain transformed cells by downregulation of MAPK signalling.

CDC25A might also contribute to cellular transformation by decreasing responsiveness to oxidative stress through downregulation of the redox-sensitive pro-apoptotic signalling kinase ASK1. CDC25A inhibits ASK1 activation by a non-catalytic protein– protein interaction that blocks ASK1 dimerization, an event that is crucial for activation of the stress-responsive kinase[77]. By overexpressing CDC25A, tumour cells might become insensitive to certain oxidative insults and acquire a growth advantage in the face of adverse stresses that would normally initiate an apoptotic programme[77]. Cells can also gain a selective growth advantage by allowing cell-cycle progression in the presence of compromised genetic material. Because CDC25A is central to the G1/S checkpoint that is initiated in response to genetic insults, overexpression of CDC25A might allow cells to circumvent anti-growth signals transmitted by CHK1 and CHK2 in response to DNA damage, proceed with genomic replication and progress through the cell cycle with damaged or altered DNA. This would increase the chance of propagating genetic abnormalities and the likelihood of acquiring a growth advantage[36,37].

CDC25B might also exert its oncogenic potential by providing cells with a selective growth advantage. CDC25B has been reported to act as a co-activator for the oestrogen, progesterone, glucocorticoid and androgen receptors in mammary glands[78]. Overexpression of CDC25B to levels that overwhelm normal physiological regulatory mechanisms might contribute to

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inappropriate cell proliferation by elevating the levels of hormone-responsive genes through CDC25B-mediated steroid-receptor transactivation[78]. As a target of cell-cycle checkpoints, CDC25B overexpression might promote mitotic entry in the presence of DNA damage by overwhelming p38-mediated anti-growth signals[60]. This would increase the likelihood of propagating genetic abnormalities and therefore increase the chances of conferring cells overexpressing CDC25B with a growth advantage.

The overexpression of members of the CDC25 family and their role in regulating cell-cycle progress and survival make them attractive targets for new, potent and selective small-molecule inhibitors. Several groups have attempted to generate inhibitors with both the selectivity and specificity for *in vivo* application, and their efforts will be detailed in the rest of this review.

Inhibitors of DSPases

Primarily because of the strong evidence indicating that CDC25A and CDC25B are potential oncogenes[63,64] and the enzymatic uniqueness of the DSPases, interest in small-molecule inhibitors of this protein class has grown. Until the mid-1990s, the only readily available DSPase inhibitor was the broad-spectrum PTPase inhibitor sodium orthovanadate. More recently, several natural products and their derivatives, as well as a number of synthetic small molecules, have been reported as inhibitors of the DSPases CDC25 and VHR . We have outlined below the reported inhibitors of CDC25 and listed in TABLES 1 and 2 some of the *in vitro* inhibitory values for selected small molecules. For many compounds, however, we have little information about their specificity against other protein phosphatases or about their efficacy against CDC25 in intact cells.

Table 2 | **Mean inhibitory concentration (IC_{50}) of compounds against protein phosphatases***

Compound‡	IC_{50} values against various protein phosphatases				
	CDC25A (μM)	CDC25B (μM)	CDC25C (μM)	VHR (μM)	PTP1B (μM)
50	1.1	–	–	–	–
51	0.9	–	–	–	–
52	0.7	–	–	–	–
53	2.1	–	–	–	–
58	–	22 ± 2	–	30 ± 1	45 ± 2
59	–	14 ± 4	–	25 ± 1	10 ± 5
60	–	20 ± 4	–	31 ± 16	20 ± 3
61	–	21 ± 4	–	20 ± 5	20 ± 8
62	–	25 ± 1	–	31 ± 1	29 ± 2
63	–	26 ± 4	–	32 ± 9	24 ± 4
64	–	72 ± 12	–	33 ± 7	85 ± 4
65	–	89 ± 11	–	32 ± 3	>100
66	–	92 ± 0	–	42 ± 1	>100
67	–	>100	–	57 ± 1	>100
68	0.7	–	–	5.0	82
69	3.0	–	–	9.0	89
70	8.0	–	–	6.1	16
71	15.0	–	–		
72	27.0	–	–		
78	–	0.21 ± 0.08	–	4.0 ± 0.1	>100
79	–	0.37 ± 0.08	–	5.1 ± 0.1	8.7 ± 0.3
80	–	0.45 ± 0.02	–	17 ± 0.5	>100
81	–	0.82 ± 0.08	–	>10	>10
82	–	0.30 ± 0.03	–		
83	–	0.59 ± 0.18	–	>10	>10
84	–	0.43 ± 0.03	–	1.1	9.8
85	–	0.13 ± 0.04	–	2.7 ± 0.55	2.3 ± 0.15
86	–	0.18 ± 0.05	–	1.8 ± 0.30	4.1 ± 1.22
87	–	0.21 ± 0.07	–	6.9 ± 1.29	>100
88	–	0.05 ± 0.13	–	5.7 ± 0.65	11.9 ± 1.58

*The inhibition of recombinant protein phosphatase was determined as described in REF 123. ‡Compound numbers relate to the numbering in the main text. The mean inhibitory concentration (IC_{50}; μM) was obtained from 3–5 measurements ± standard error of mean (SEM). Values that lack ± are from a single determination. – indicates that the value has not been determined. CDC, cell-division cycle; PTP1B, protein tyrosine phosphatase 1B; VHR, vaccinia virus phosphatase VH1-related.

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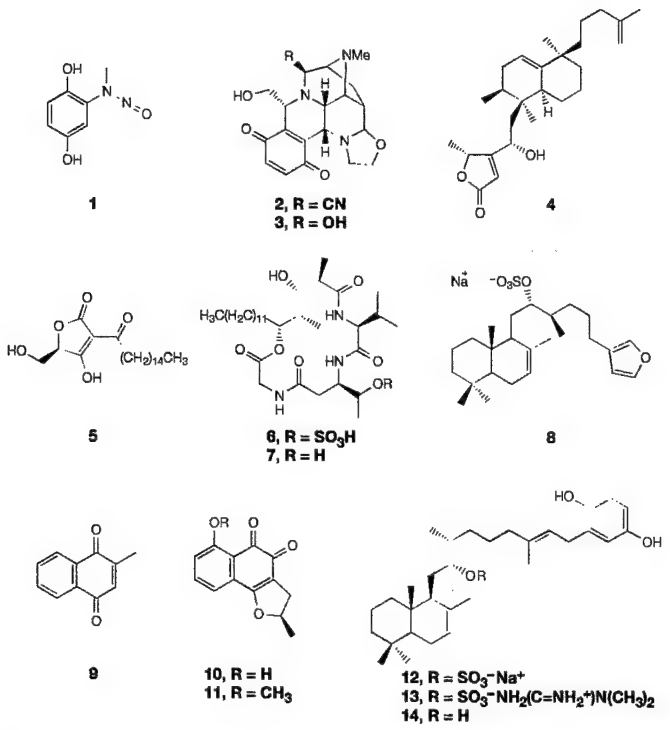

Figure 4 | **Natural-product inhibitors.**

Natural-product inhibitors

Dephostatin (compound 1 in FIG. 4), a 2,5-dihydroxy nitrosoaniline, was isolated from the *Streptomyces* strain MJ724-NF5 and identified as an active compound in a screen for inhibitory activity against PTPases[79]. This compound caused irreversible deactivation of the enzyme. Although this natural product was screened specifically against PTPases, unpublished results from studies carried out in the Eckstein laboratory indicated that dephostatin had similar activity against the catalytic domain of CDC25 (REF. 80).

The benzoquinone antitumour antibiotics dnacin A1 (compound 2) and B1 (compound 3) were isolated from the *Nocardia* strain C-14482 (N-1001) and screened initially for antibiotic activity[81]. They were found to interact with DNA in susceptible cells. A DNA-bound dnacin B1 complex generated oxygen free radicals that were responsible for eventual DNA damage and cell death. These benzoquinones were later screened against glutathione-*S*-transferase (GST)-tagged– CDC25B, and were found to inhibit the enzyme with modest half-maximal inhibitory concentration (IC_{50}) values of 141 and 64.4 μM, respectively[82]. Kinetic analysis indicated that the dnacins inhibited CDC25B in a non-competitive manner (inhibition constant, K_i = 0.16 and 0.10 μM, respectively). It has been postulated that the inhibition might be effected by covalent modification of the enzyme by a 1,4-MICHAEL-TYPE NUCLEOPHILIC ADDITION[81].

MICHAEL-TYPE NUCLEOPHILIC ADDITION
An addition of a new substituent to a double bond that is conjugated to an electron-accepting functional group.

Subsequent to the disclosure on dnacins, another DSPase inhibitor, the sesquiterpene γ-hydroxybutenolide dysidiolide (compound 4), was isolated from the Caribbean sponge *Dysidea etheria*. Dysidiolide was shown to inhibit CDC25A, thereby preventing the dephosphorylation of *para*-nitrophenol phosphate (*p*-NPP) with an IC_{50} of 9.4 μM (REF. 83). The natural product was also shown to inhibit the growth of A-549 human-lung adenocarcinoma and P388 mouse-leukaemia cell lines with IC_{50} values of 4.7 and 1.5 μM, respectively. The γ-hydroxybutenolide might act as a phosphate surrogate. Surprisingly, a screen by Pal and colleagues[84] found that both the natural and synthetic γ-hydroxybutenolide dysidiolide and several analogues were inactive against GST–CDC25A when *O*-methyl fluorescein monophosphate (OMFP) was used as a substrate. However, later, Shirai and co-workers in their studies of the natural product as well as dysidiolide analogues, again indicated that the natural product was moderately active against CDC25A (IC_{50} = 35 μM when *p*-NPP was used as substrate)[85]. It is unclear whether the reported differences reflect the importance of the substrate in the assay.

The hexadecanoyl-5-hydroxymethyl tetronic acid RK-682 (compound 5) was isolated from the *Streptomyces* strain 88-682 and was found to be a comparatively potent non-competitive inhibitor of the DSPase VHR, with an IC_{50} of 2.0 μM (REF. 86). RK-682 also caused the arrest of cell-cycle progression at the G1 checkpoint in mammalian cells. Similar to the benzoquinones (compounds 2 and 3), RK-682 might also interact with the enzyme by a 1,4-Michael-type nucleophilic addition, resulting in a covalent modification of the enzyme.

Two immunosupressant cyclic depsipeptides, stevastelins A (compound 6) and B (compound 7), were isolated from the *Penicillium* strain NK374186 and shown to be good inhibitors of VHR, with IC_{50} values of 2.7 and 19.8 μM, respectively[87,88]. Stevastelin A, although potently inhibiting VHR in extracellular enzyme preparations, showed very little effect on cellular preparations. The converse was true of stevastelin B. This could be attributed to the poor permeability of stevastelin A, whereas stevastelin B, which lacks the threonyl sulphate, traverses the cell membrane and is activated intracellularly, presumably by either sulphation or phosphorylation at the threonyl hydroxyl.

Sulfircin (compound 8), a bicyclic, sulphated sesquiterpenoid with an aliphatic chain terminating in a furan, was isolated by Wright and co-workers in 1989 from a deep-water sponge of the genus *Ircinia*[89]. This marine natural product and analogues thereof were investigated for their phosphatase inhibitory activity against a panel of PTPases. Sulfircin was found to inhibit CDC25A with an IC_{50} of 7.8 μM and VHR with an IC_{50} of 4.7 μM (REF. 90).

Vitamin K_3, (menadione; compound 9) was shown to have antiproliferative activity towards a wide range of human cells. It also enhanced the antiproliferative effects of other clinically useful anticancer agents, and had lower levels of toxicity to animals compared with other

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quinone-type chemotherapeutic agents[91]. Menadione was found to inactivate CDC25B with an IC_{50} of 3.6 ± 0.6 μM in an irreversible manner by covalently binding at or near the active site[92,93].

Two tricyclic polyketide *ortho*-quinone antibiotics, nocardinones A (compound **10**) and B (compound **11**), were isolated from the fermentation broth of the *Nocardis* strain TP-A0248 and were found to inhibit the activity of the CDC25B DSPase with an IC_{50} of 17 μM (REF. 94). These *ortho*-quinones were, however, not selective for CDC25B, as they were similarly active against protein tyrosine phosphatase 1B (PTP1B; IC_{50} = 14 μM). However, they emerged as potent cytotoxic substances, inhibiting the growth of human cervical carcinoma HeLa and human lung cancer SBC-5 cell lines with IC_{50} values of 0.38 μM and 0.54 μM, respectively.

The sesquiterpene sulphate coscinosulphate (compound **12**) and its dimethyl guanidine analogue (compound **13**) were isolated from the New Caledonian marine sponge *Coscinderma mathewsi* in a bioassay-directed isolation protocol. In addition to its proven antimicrobial activity towards *Staphylococcus aureus*, compound **12** was a potent deactivator of CDC25A (IC_{50} = 3.0 μM). Compound **13** was less potent, with an IC_{50} of 18 μM. The hydrolysed sesquiterpenoid (compound **14**; IC_{50} = 30 μM) that was prepared during the process of structural elucidation was significantly less potent than compounds **12** and **13**. This result indicated that the sulphate function might be necessary for the inhibitory activity of this group of natural products[95].

ARYLATION
The attachment of an aromatic substituent to an organic residue.

FLOW CYTOMETRY
A multi-parametric method in which suspended cells flow through a chamber and can be counted and analysed for size and content of fluorescence-tagged components.

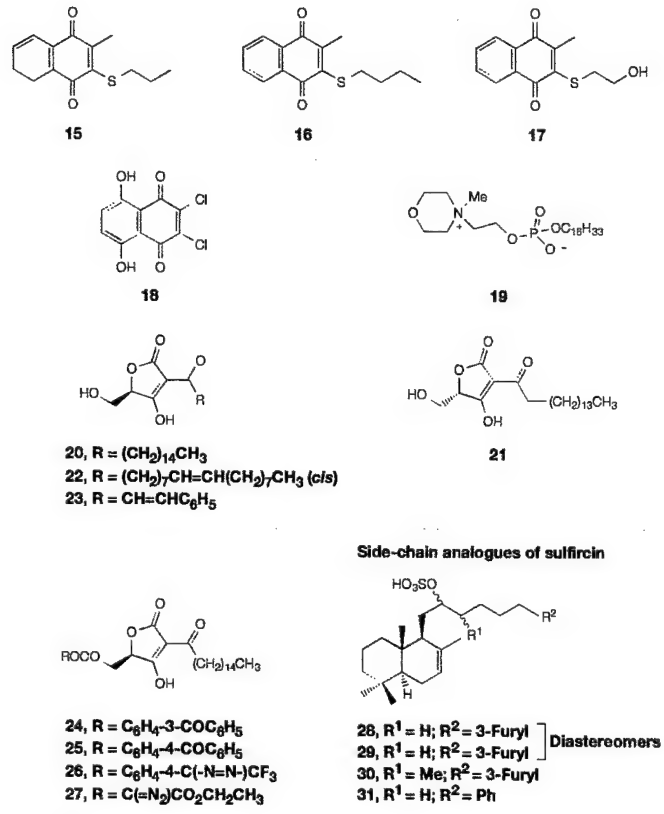

Figure 5 | **Natural-product analogues.** Me, methyl group; Ph, phenyl group.

Natural-product analogues

In an effort to improve the activity of some of the reported natural inhibitors, or to explore new lead structures, several investigators prepared synthetic derivatives of the natural products. These substances have also assisted in the elucidation of some of the structural features that are necessary for inhibition of the protein.

Several synthetic vitamin K derivatives reported by Nishikawa and co-workers[96] were examined for their growth inhibitory activities against hepatoma cells. Of this series of analogues, the 3-thioether-containing naphthoquinones (compounds **15–17** in FIG. 5) were found to be the most potent. Compound **17**, which contains a 3-thioethanol side chain, inhibited hepatoma cell growth with a 50% growth inhibitory dose (ID_{50}) of 5.6 μM. A series of experiments by Nishikawa *et al.* established that the mechanism of action involved direct ARYLATION of cellular thiols by the electrophilic naphthoquinones. Lazo and colleagues[93] later established that compound **17** was a time-dependent inhibitor of the CDC25 phosphatases, with selectivity for CDC25A (IC_{50} = 3.8 μM). Compound **17** arrested cells in both the G1 and G2/M phases, as shown in FLOW CYTOMETRIC studies. It is possible that sulphydryl arylation of the catalytic cysteine of the DSPase binding pocket (FIG. 1) is responsible for the inactivation of the proteins, even though there are five cysteines present in the catalytic domain of CDC25B[28].

Ham *et al.*[97] speculated that the hydrophobicity of compound **9** and its thioether analogue compound **17** would lead to physico-chemical interactions with the cell membrane, resulting in cytotoxicity. Therefore, they examined the potency of a group of more hydrophilic naphthoquinone derivatives from commercial and synthetic sources[97]. According to their postulate, the introduction of hydroxyl groups at the C-5 or C-8 positions would increase water solubility. Furthermore, stabilization by hydrogen bonding between the hydroxyl group and the developing enolate anion would increase the ability of the substrate to undergo Michael-type additions. Halogen substituents at the C-2 and C-3 positions would also make the carbon atoms more electrophilic and susceptible to thiolate addition, whereas the elimination of the halide ion would promote the irreversibility of the inactivation. This postulate was supported by the observation that 2,3-dichloro-5,8-dihydroxynaphthoquinone (compound **18**) was the most active of the analogues screened. Flow cytometry analysis showed a cell-cycle delay at the G1/S phase transition, supporting a decrease in the CDC25A phosphatase activity caused by compound **18**.

Ether lipids and alkylphospholipids have shown efficacy against malignant cell lines both *in vitro* and *in vivo*. Two series of analogues of alkylphospholipids

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were synthesized, bearing *N*-methylmorpholino or *N*-methylpiperidino head groups with long aliphatic or alkoxyethyl chains. They were evaluated for their cytotoxic activity *in vitro* against a panel of human xenografts and their ability to inhibit CDC25 phosphatase, and were shown to cause very weak inhibition of DSPases. The most active derivative was the *N*-morpholino derivative (compound **19**); IC_{50} = 40 μM), which showed a decrease in potency compared with hexadecylphosphocholine, which had an IC_{50} of 25 μM (REF. 98).

Asymmetric syntheses of RK-682 (compound **5**) and its analogues (compounds **20–22**) provided substances that maintained selectivity and potency towards VHR (IC_{50} = 1.0–3.4 μM). Sodeoka *et al.*[99] showed that the stereochemistry of the C-5 substituent was not important for activity — the synthetic (*R*)- (compound **20**) and (*S*)- (compound **21**) C-5 isomers, as well as the natural product, showed similar activities against VHR. Compound **23**, which has a cinnamoyl side chain at C-3, was completely inactive, whereas the long-chain *cis*-alkene compound **22** showed moderate inhibition of CDC25A and B (IC_{50} = 34 μM). This indicated that the C-3 substituent might be important for enzyme recognition.

Further derivatization of RK-682 by Sodeoka's group by manipulating the substituents at C-3 and increasing the hydrophobicity at the C-5 position resulted in potent inhibitors with selectivity for CDC25B[100]. Using the methodology previously developed for the asymmetric synthesis of RK-682 and compound **21**, further analogues were prepared. The most potent of these, compounds **24–27**, were obtained by acylation of RK-682 and other advanced tetronic-acid derivatives. Although they are still showing good inhibition of VHR, compounds **24–27** also showed some 30-fold or more increased selectivity for CDC25B[100].

Bockovich and colleagues[90] prepared 24 analogues of sulfircin (compound **8**) to probe the importance of its three core structures — namely, the sesquiterpenoid tricycle, the alkylfuryl moiety and the sulphate group — for inhibitory activity. The most active analogues, compounds **28–31**, are shown in FIG. 5 (REF. 90). This study revealed that the length of the aliphatic chain was crucial; compounds with the longest side chain were equipotent or more potent than the natural product. Although the sulphate group was important for activity, the stereochemistry of the sulphate was not important (compound **28** compared with compound **29**). Replacement of the sulphate with a malonate group also provided analogues that were equipotent to sulfircin. The presence or absence of the C-12 methyl group, or replacing the furyl substituent with a phenyl ring, had little or no effect on the potency of these analogues. Replacement of the sesquiterpenoid core with a benzimidazole, benzothiazole or naphthyl ring system, however, rendered these compounds completely inactive[90].

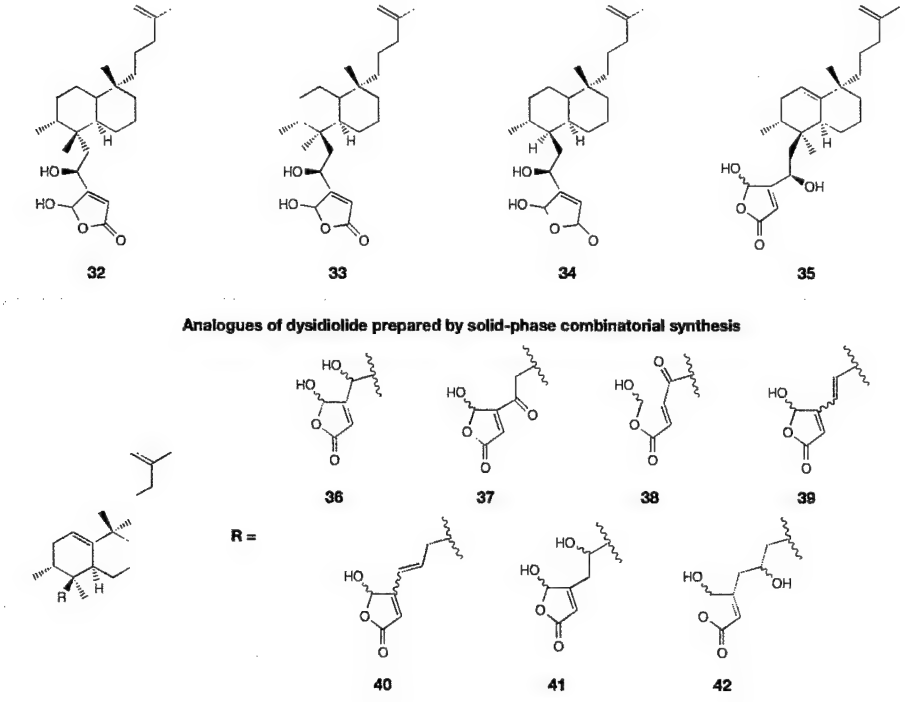

Figure 6 | **Natural-product analogues.**

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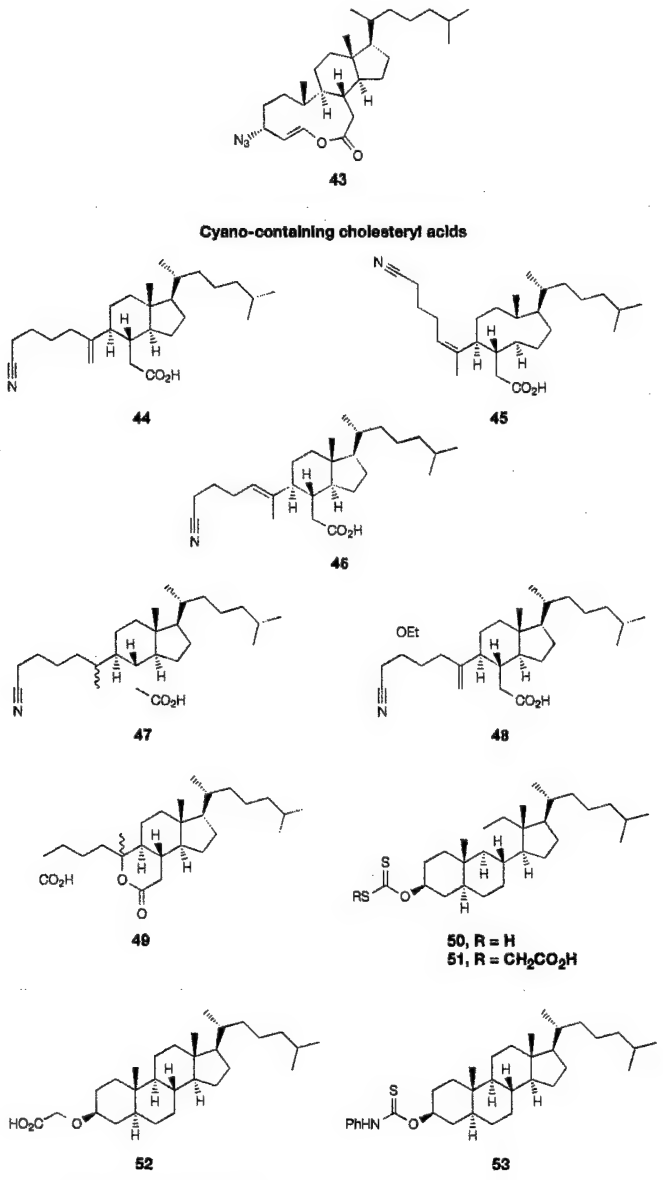

Figure 7 | **Natural-product analogues.**

Since the initial report of its structure and DSPase activity, dysidiolide (compound 4) has been the subject of many asymmetric and racemic total syntheses[101–109]. Various analogues have also been prepared (FIG. 6), and their potencies have been compared with the natural product. Shirai and co-workers[85] synthesized 4-*epi*-dysidiolide (compound 32) and 4,6-bis-*epi*-dysidiolide (compound 33), as well as the 6-desmethyl compound 34. Compounds 32–34 inhibited CDC25A and B at lower concentrations than the natural product (IC_{50} = 13–15 μM with *p*-NPP, compared with 35 μM)[85]. The epimer (compound 33) was not only a more potent inhibitor of the protein, but it also inhibited the growth of the human lung-cancer cell line SBC-5 and the human leukaemia cell line HL60 at IC_{50} values of 1.3 and 1.0 μM, respectively (compared with IC_{50} values of 5.4 and 7.1 μM, respectively for dysidiolide).

A combinatorial solid-phase approach towards the design of dysidiolide derivatives was used by Waldmann and co-workers[110]. They synthesized 6-*epi*-dysidiolide (compound 35) and a series of analogues with structural variations between the γ-hydroxybutenolide and the bicyclic core. 6-*epi*-Dysidiolide (compound 35), as well as the derivatives (compounds 36–42), were shown to inhibit CDC25C with IC_{50} values of 0.8–16 μM (FIG. 6). Replacing the hydroxyethyl bridge between the bicyclic core and the hydroxybutenolide with either an unsaturated three-carbon fragment or a keto group resulted in compounds that were more potent than the natural product against CDC25C. Most notable is the activity of the ketone compound 37 (IC_{50} = 800 nM for CDC25C). The epimer compound 35 showed some selectivity for CDC25C, with an IC_{50} of 5.1 μM compared with 13 and 18 μM for CDC25A and CDC25B, respectively. Although the ketone compounds 37 and 38 were more potent inhibitors of the phosphatase, they were far less cytotoxic than compound 35, the alcohol compound 36 and the alkene compounds 39 and 40.

It has been suggested that in dysidiolide, the γ-hydroxybutenolide moiety probably functions as a phosphate mimic, whereas the long side chain occupies a hydrophobic binding pocket near the active site. With this information, Zalkow and co-workers fashioned a new class of CDC25A inhibitors based on steroidal templates[111–113]. They reasoned that the rigidity and well-defined stereochemistry of the cholesteryl rings should provide a good scaffold for distal functionalization with suitable phosphate surrogates and hydrophobic side chains. Transformation of cholesteryl acetate to 3-α-azido-B-homo-6-oxa-4-cholesten-7-one (compound 43) and subsequent silica-gel-supported pyrolysis led to a wide range of rearranged products that arise presumably by an azide-mediated fragmentation mechanism. Further synthetic manipulations of some of these novel rearrangement products provided quite potent inhibitors of human CDC25A, which are shown in FIG. 7[111]. Compound 44, one of the best inhibitors in this class, inhibited the activity of CDC25A reversibly and non-competitively with an IC_{50} of 2.2 μM. These results indicated that these compounds were possibly interacting with the enzyme through an arginine group at a site distinct from the active site[111,113]. The carboxylic-acid derivative compound 49, resulting from base hydrolysis of the saturated nitrile lactone, was the most potent of the modified lactone-pyrolysis products (IC_{50} = 5.1 μM for CDC25A)[113].

A series of cholestanol derivatives was also synthesized to evaluate alternative phosphate surrogates[112]. With standard chemical procedures, the xanthate, carboxyl and thiocarbamate functionalities were introduced. The most

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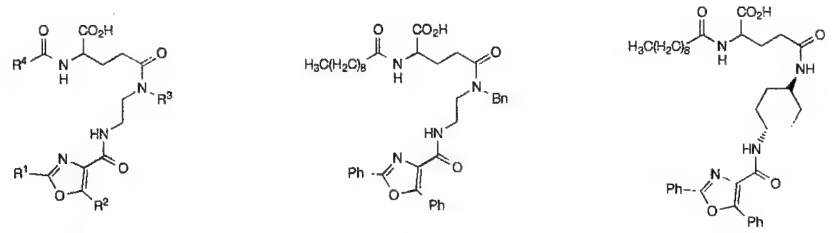
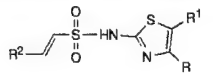


54 55 56

Structures of sulphonylated aminothiazole inhibitors of protein phosphatases



57

58, R = Ph; R^1 = Ph; R^2 = 2-Naphthyl
59, R = (4-CF_3)Ph; R^1 = *n*-Propyl; R^2 = (2-Cl)Ph
60, R = (4-CF_3)Ph; R^1 = *n*-Propyl; R^2 = (4-Me)Ph
61, R = (4-Cl)Ph; R^1 = *n*-Propyl; R^2 = (3,4-diCl)Ph
62, R = (4-CF_3)Ph; R^1 = *n*-Propyl; R^2 = (3,4-diF)Ph
63, R = (4-Cl)Ph; R^1 = *n*-Propyl; R^2 = (2-Cl)Ph
64, R = Ph; R^1 = Ph; R^2 = (3,4-diF)Ph
65, R = Ph; R^1 = Et; R^2 = 2-Naphthyl
66, R = (4-Ph)Ph; R^1 = *n*-Propyl; R^2 = (4-CF_3)Ph
67, R = (4-Ph)Ph; R^1 = *n*-Propyl; R^2 = 2-Naphthyl

Figure 8 | **Synthetic inhibitors.** Et, ethyl group; Me, methyl group; Ph, phenyl group.

active among this set of analogues, compounds **50–53**, inhibited CDC25A with IC_{50} values of 0.7–2.1 μM. Several of these analogues were shown to have antiproliferative activity in the National Cancer Institute (NCI) 60-cell-line human tumour panel (see NCI/NIH Developmental Therapeutics Program online).

Synthetic inhibitors

A basic pharmacophore model was designed by Wipf *et al.*[114] on the basis of structure–activity relationship (SAR) data that were available for the natural-product inhibitors of PS/TPases. A total of 18 analogues based on compound **54** were synthesized by a solid-phase combinatorial approach (FIG. 8). One member of the library, 4-(benzyl-(2[(2,5-diphenyl-oxaole-4-carbonyl)-amino]-ethyl)-carbomyl)-2-decanoylamino butyric acid (SC-ααδ9; compound **55**), emerged as the most potent competitive inhibitor of both CDC25A and B, with an IC_{50} of 15 μM, while inhibiting PTP1B non-competitively[115]. The inhibition of CDC25 protein phosphatases was further substantiated by cell-growth and cell-cycle progression studies. Compound **55** selectively inhibited the growth of mouse embryonic fibroblast cells transformed with the Simian virus 40 (SV40) large T antigen, a model of a malignant phenotype that overexpresses CDC25B. Flow cytometry indicated inhibition at the G1 and G2/M phases in the presence of compound **55**, as might be predicted from our understanding of the role of CDC25 in regulating the phosphorylation status and, therefore, the activity of several CDKs (FIG. 3). Moreover, increased phosphorylation of CDK1, CDK2 and CDK4 was detected, consistent with inhibition of intracellular CDC25 activity. Compound **55** was found to be cytotoxic to human breast-carcinoma cells (MDA-MB-231) with an IC_{50} of <100 μM (REF. 116).

After the identification of compound **55**, careful examination of the variable regions of this molecule revealed that the hydrophobicity of the side chain and the presence of an aromatic moiety at C-5 of the oxazole (R^4 in compound **54**) were crucial for CDC25B inhibitory activity. Replacing the ethylenediamine moiety of the core region with cyclohexylamine provided a new inhibitor, FY21-αα09 (compound **56**; REF. 117). Although compound **56** had inhibitory activity against PTP1B (IC_{50} = 41.4 μM), it was approximately fourfold more active against CDC25B2 and VHR (IC_{50} values of 7.0 μM and 12.1 μM, respectively). The kinetics of CDC25B2 inhibition indicated that FY21-αα09 is a partial competitive inhibitor (K_i = 1.6 ± 0.2 μM). Compound **56** caused >90% inhibition of growth of breast-cancer cell lines MDA-MB-231 and MCF-7 at 100 μM. It also blocked cell-cycle progression at the G2/M checkpoint, an observation consistent with intracellular inhibition of CDC25B2.

The SAR observed by the influence of substituents at the oxazole moiety of SC-ααδ9 led to the development of a new heterocyclic scaffold, compound **57**. A library of these sulphonylated aminothiazoles was prepared and screened for inhibitory activity against CDC25B, VHR and PTP1B[118]. Of this 35-compound library, 15 compounds had IC_{50} values for CDC25B <50 μM and 5 had IC_{50} values ≤25 μM. Among the best inhibitors, with the exception of compound **58**, compounds **59–63** were all substituted with halogenated aromatic rings at positions R and R^2 (FIG. 8). However, unlike the parent pharmacophore SC-ααδ9, with the favoured diphenyl oxazoles (except for compound **58**), these more active aminothiazoles had *n*-propyl moieties at R^1. Of this small library of thiazoles, compound **63** was found to inhibit CDC25B with a K_i of 4.6 ± 4 μM, which was

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below that determined for SC-ααδ9. Consistent with the signature motif of the active site of CDC25B, VHR and PTP1B, almost all the compounds that were inactive against CDC25B were also inactive against VHR and PTP1B. However, although lacking significant inhibitory activity against CDC25B and PTP1B, compounds **64–67** retained moderate activity against VHR.

The use of phosphate surrogates has already been discussed for some of the natural-product derivatives. Pal and co-workers[119] synthesized and evaluated a group of dipeptide mimics containing established phosphate surrogates. It was predicted that the phosphate surrogate would anchor the compounds in the active site, whereas the core structure would probe the other necessary binding interactions within the binding cavity. By using these established phosphate mimics as reactants in a four-component UGI REACTION, these surrogates could be positioned at either the amino terminus or in the centre of the peptidic compound. This strategy would allow varied regions of the active site to be interrogated. Three distinct libraries were prepared, including one in which both a phosphorylated acid and an aldehyde were used as Ugi-reaction components. A total of 4,320 members were synthesized and screened as crude mixtures against CDC25A. Active mixtures were then resynthesized, purified and screened against CDC25A, VHR and PTP1B. Nine members of the library emerged as good inhibitors, with IC_{50} values ranging from 0.7 to 35 μM and 5.0 to 87 μM for CDC25A and VHR, respectively. The most potent members of this library were compounds **68–70** (FIG. 9). With the exception of compound **69**, the phosphate surrogate is in the former aldehyde component in the centre of the peptide mimic. Mechanistic analysis indicated, however, that these Ugi products are non-competitive inhibitors. This was evidenced by the fact that analogue **71**, which lacked a phosphate mimic, and compound **72**, which contains an α,α-difluoromethylenephosphonate moiety that should provide improved binding over methylenephosphonates, showed equivalent activity against CDC25A (IC_{50} values of 15 and 27 μM, respectively).

The screening of a collection of compounds at Pharmacia & Upjohn for inhibitory activity against CDC25B provided the weakly active lead compound PNU-108937 (compound **73**; IC_{50} = 70–100 μM). A solid-phase library of 24 tetrahydroisoquinolines that were based on this lead was prepared from commercially available 3,5-di-iodo-L-tyrosine using a PICTET-SPENGLER CYCLIZATION[120]. The initial library provided

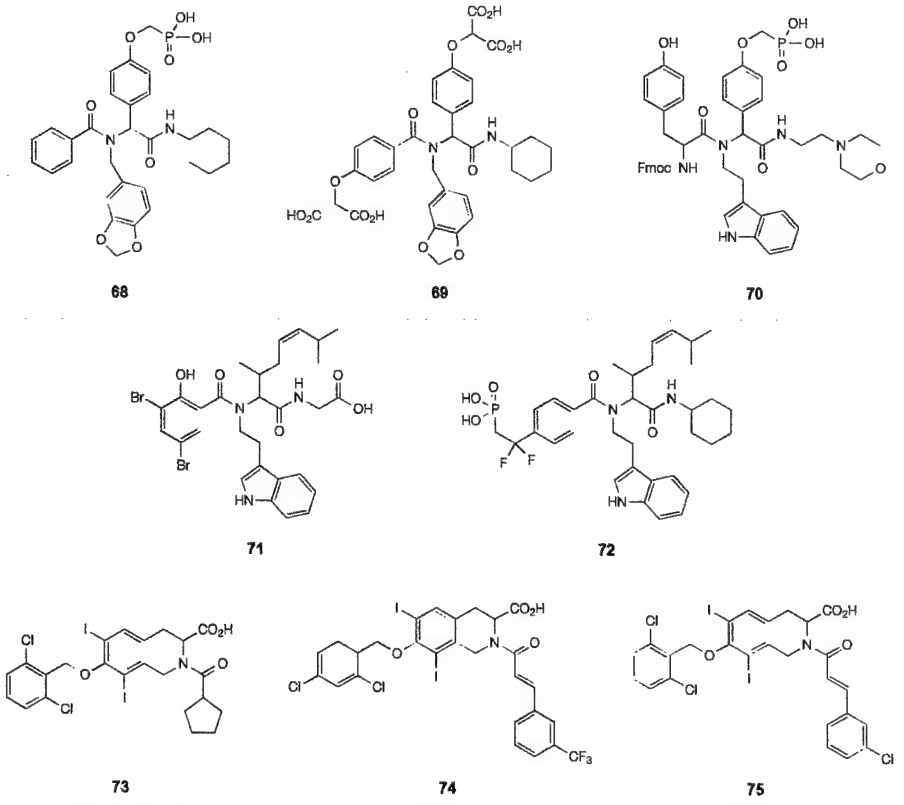

Figure 9 | **Synthetic inhibitors.**

UGI REACTION
The condensation reaction of an amine, an isocyanide, an aldehyde and an acid to form a peptide-like strand.

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compounds that were equipotent to the lead. Among these, compounds **74** and **75** had good inhibitory activity against CDC25B, with IC_{50} values of 15–20 μM. A second library of 51 members was generated, bearing various cinnamoyl substituents at N-2 and alkyl substituents at the C-7 phenol. However, this second series showed no improved potency over the parent compounds.

Although the 8-phenylthioquinolinone carboxylic acid compound **76** and its naphthyridinone derivative compound **77** have been reported to inhibit CDC25 phosphatase with IC_{50} values of 11 μM and 5 μM, respectively[121], a more potent series of heterocyclic diones was recently discovered (FIG. 10). The screening of the NCI Diversity Set — which contained 1,990 compounds selected as representative of the entire 140,000 NCI compound library — by Lazo *et al.*[122] resulted in the identification of eight potent quinolinediones, and of these, the 7-substituted quinolinediones were the most potent. All submicromolar inhibitors showed a strong preference for CDC25B compared with VHR or PTP1B.

NSC 663284 (compound **78**), the most potent compound, inhibited CDC25B and VHR with IC_{50} values of 206 ± 75 nM and 4.0 ± 0.1 μM, respectively. Inhibition kinetics for each of the full-length human CDC25 isoforms indicated that compound **78** most closely fitted a partial mixed-inhibition model. This might reflect the potential for interaction at the two anionic binding sites as observed in the crystal structure of CDC25B[112]. The K_i values indicated that NSC 663284 had a threefold selectivity towards CDC25A compared with CDC25B or CDC25C. NSC 663284 had a mean IC_{50} value in the NCI 60-cell-line human tumour panel of 1.5 ± 0.6 μM when the cells were treated for 48 hours. The most sensitive cell lines were human breast cancer MDA-MB-435 and MDA-N cells (IC_{50} = 0.2 μM). The synthesis of compound **78**, its REGIOISOMER (compound **81**) and analogues (compounds **82–84**), attested to the observation that the 7-substituted quinolinediones were more potent than the 6-substituted derivatives or the 6,7-dichloro- intermediates. The 2-morpholin-4-ylethylamino moiety seems to be one of at least two unique enhancers of activity.

Another set of inhibitory substances was identified from an *in vitro* screening of the NCI's chemical substances against oncogenic, full-length, recombinant human CDC25B. Twenty-one compounds were found to have mean inhibitory concentrations of <1 μM (REF. 123). Four of these — NSC 95397, 139049, 135880 and 115447 (compounds **85–88**) — had IC_{50} values for CDC25B of less than 500 nM. NSC 95397, the most potent DSPase inhibitor to date, had IC_{50} values of 125 ± 36 nM, 22.3 ± 5.9 nM and 56.9 ± 17.7 nM for CDC25B, A and C, respectively, and showed partial mixed-inhibitory kinetics. Compound **85**, in an initial evaluation with the entire NCI 60-cell-line human

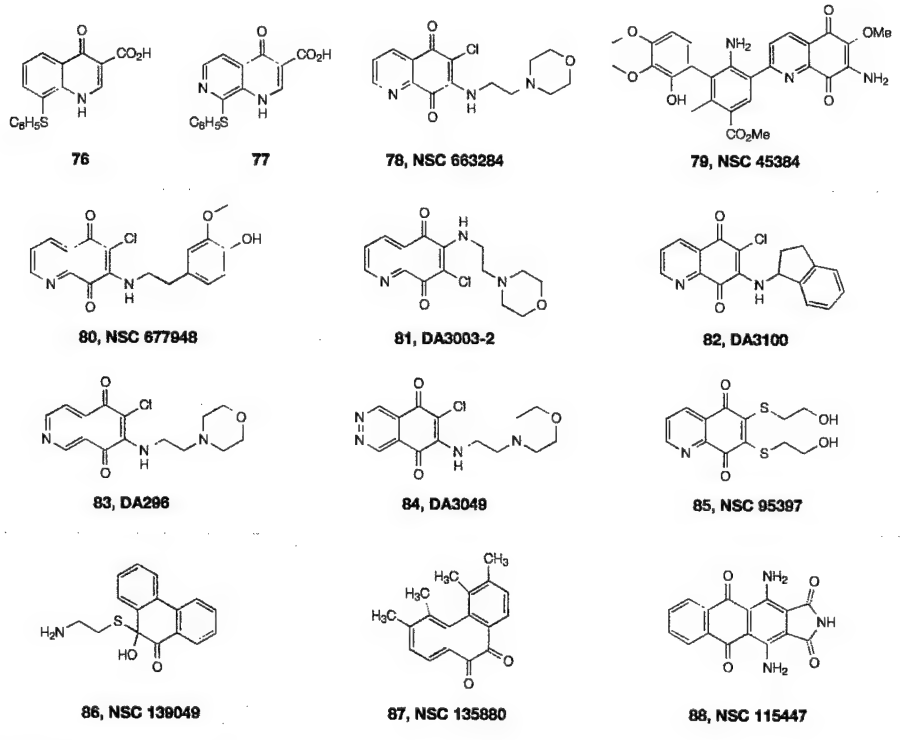

Figure 10 | **Synthetic inhibitors.**

PICTET–SPENGLER CYCLIZATION
The cyclocondensation of an aromatic ethylamine and an aldehyde or ketone to give a tetrahydroisoquinoline.

REGIOISOMERS
Compounds that differ in the arrangement of substituents.

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tumour panel, showed an overall mean growth inhibitory concentration of 1 μM. MOLT-4 leukaemia, LOX IMVI melanoma and SK-MEL-5 melanoma were the most sensitive cell lines.

Summary

DSPases represent an unusually highly regulated subclass of PTPases. Clearly, the CDC25 members are attractive targets for inhibition, as they have been implicated in cancer and Alzheimer's disease. A more comprehensive understanding of the physiological context in which the DSPases function is desirable. Selective and potent small-molecule inhibitors of the catalytic activity would not only supplement standard genetic approaches to studying CDC25 function but would also provide valuable tools for both reversible and graded enzyme inhibition. Although most of the current inhibitors lack either the requisite potency or selectivity to be ideal, the diverse structural architecture of recently identified compounds offers hope that improved inhibitors can be identified.

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Regulation of Cdc25A Half-Life in Interphase by Cyclin-Dependent Kinase 2 Activity

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Summary

Cdc25A regulates cell cycle progression, has oncogenic and anti-apoptotic activity, and is overexpressed in many human tumors. Phosphorylation by Chk1 and Cds1/Chk2 downregulates Cdc25A levels in response to genotoxic stresses. Nevertheless, it remains unclear whether Chk1 and Cds1/Chk2 are uniquely responsible for regulating Cdc25A stability during interphase or if other kinase activities contribute. Here we report that treatment of HeLa cells with the cyclin-dependent kinase inhibitor roscovitine caused a concentration- and time-dependent increase in Cdc25A protein levels. Transfection with dominant-negative cdk mutants demonstrated that only a Cdk2 mutant increased Cdc25A protein levels; Cdk1 and Cdk3 mutants had no effect. The increased Cdc25A protein levels were the result of an increase in the half-life of the protein; no increase in Cdc25A mRNA levels was observed. These results demonstrate Cdk2 kinase activity contributes to the labile nature of Cdc25A during interphase and redefine the nature of the Cdc25A-Cdk2 autoamplification feedback loop.

Introduction

The Cdc25 dual-specificity phosphatases catalyze cell cycle progression by dephosphorylating and activating the Cyclin-dependent kinases (Cdk)[1]; the three human *cdc25* homologs, Cdc25A, Cdc25B, and Cdc25C, regulate different phases of the cell cycle. Most investigators believe that Cdc25C functions primarily in mitosis and catalyzes mitotic progression by activating Cdk1/Cyclin B, as Cdc25C is the human Cdc25 isoform most homologous to yeast and Xenopus Cdc25 (1-4). Cdc25B was originally characterized as functionally redundant to Cdc25C due to its ability to activate Cdk1/Cyclin B (5). More recently, Cdc25B has been implicated as the activator of Cdk1/Cyclin B at the onset of mitosis and of Cdk2/Cyclin A in late G2 and the target of a Chk1- and Cds1/Chk2-independent G2/M checkpoint (6-8). Since the primary Cdk substrate for Cdc25A seems to be Cdk2/Cyclin E, Cdc25A was relegated to promoting the G1/S cell cycle transition and S phase progression (9, 10). Cdc25A protein levels and activity, however, remain present past S phase and seem to increase as cells enter mitosis (9, 11). It has been recently reported that Cdk1/Cyclin B-mediated phosphorylation of Cdc25A increases its stability in mitotic cell populations, further supporting a role for Cdc25A in mitosis, although the functional significance of elevated Cdc25A activity throughout G2 and mitosis remains unclear (12).

An essential cellular alteration for malignant transformation is deregulation of cell cycle control proteins (13). Indeed, overexpression of Cdc25A has been reported to transform normal mouse embryonic fibroblasts in cooperation with an

oncogenic isoform of Ras (Ha-Ras^{G12V}) or in an Rb$^{-/-}$ background (14) and Cdc25A overexpression has been documented in numerous human cancers (15). The oncogenic activity of Cdc25A can be attributed to its impingement on several signaling pathways regulating cell cycle checkpoints, growth factor and hormonal mitogenesis, apoptosis and senescence (15, 16).

Because the oncogenic potential of Cdc25A is potentially dependent on both its abundance and its catalytic activity, the mechanisms regulating Cdc25A activity and expression level are of considerable interest. Transcriptional regulation of the Cdc25A promoter has been attributed to both E2F and c-myc transcription factors and seems to be cell cycle dependent, with increases in Cdc25A mRNA occurring predominantly prior to S phase, consistent with its essential role in S phase induction (9, 10, 17-19). Post-translational modification of Cdc25A has both positive and negative effects on its activity and protein levels. Cdc25A protein levels are tightly regulated by proteasome-mediated degradation pathways that may involve multiple ubiquitin ligases (11, 20-23). Cdc25A is phosphorylated by Cdk2/Cyclin E in a positive feedback loop, which increases the activity of both proteins sufficiently to cross the threshold required for the G1/S transition (24, 25). Cdc25A-activating phosphorylations have also been attributed to Raf-1 and Pim-1 kinases (26, 27). While the phosphorylation of Cdc25A by Cdk1/Cyclin B has not been reported to increase its phosphatase activity per se, it does lead to increased protein stability in mitotic cell populations (12). On the other hand, Cdc25A protein stability is negatively regulated in a cell cycle checkpoint-dependent manner by phosphorylation at serine 123, enabling

poly-ubiquitination and subsequent degradation (11, 21, 22). However, the mechanisms regulating Cdc25A protein stability in the absence of genetic insults remain unclear.

Due to the highly labile nature of Cdc25A protein, we hypothesized that a candidate for interphase regulation of Cdc25A protein levels in the absence of genetic insults would be a proximal downstream effector. By analogy, the stability of Cdc25B is decreased following phosphorylation by one of its proximal downstream effectors, Cdk1/Cyclin A (28). To explore the possibility that cdk-mediated phosphorylation of Cdc25A contributes to its inherent instability in interphase, we treated HeLa cells with the cdk inhibitors roscovitine or olomucine and found that inhibition of cdk activity resulted in a concentration- and time-dependent increase in Cdc25A protein levels. Because of the selectivity profile for roscovitine and olomucine, we employed dominant-negative cdk mutants to determine which cdk activity contributed to altered Cdc25A protein levels. Cdc25A protein levels were uniquely increased by a dominant-negative Cdk2 mutant; no increase was seen with either dominant-negative Cdk1 or Cdk3 mutants. The increased Cdc25A protein levels appeared to be the result of an increase in the half-life of the protein and no increase in Cdc25A mRNA levels was observed. These results support the hypothesis that Cdk2 kinase activity contributes to the labile nature of Cdc25A during interphase and describe how Cdc25A protein levels can be maintained under strict control until increased protein levels are necessary as cells approach mitosis.

Experimental Procedures

Reagents: Roscovitine, olomucine and cycloheximide were purchased from Calbiochem (La Jolla, California). Plasmids expressing dominant-negative (DN) mutants of Cdk1, Cdk2 and Cdk3 were generously provided by Dr. Sander van den Heuvel (29). Lipofectamine PLUS™ was from Invitrogen (Carlsbad, CA). Primary antibodies specific for Cdc25A (F6), Cdc2 p34 (17), Cdk2 (M2), Cdk3 (Y-20) and vinculin (H-300) were from Santa Cruz (Santa Cruz, CA), Cdc25B antibodies were from BD Transduction Labs (Lexington, KY) and β-tubulin antibodies were from Cedarlane Laboratories (Hornby, Ontario). Peroxidase-conjugated goat-anti-mouse and goat-anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Digoxigenin (DIG)-labeled RNA Molecular Weight Marker I, DIG-labeled actin RNA probe, PCR DIG Probe Synthesis Kit, Anti-DIG-AP Fab fragments, CDP-*Star* ultra-sensitive chemiluminescent substrate for AP, and DIG Wash and Block Buffer set were from Roche Applied Science (Indianapolis, IN).

Cell culture: HeLa human cervical carcinoma cells (HPV-positive) and MCF-7 human mammary adenocarcinoma cells (American Tissue Culture Collection, Manassas, VA) were maintained in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin-streptomycin (Gibco/Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO_2 at 37°C.

Western Blotting: Cells were harvested and lysed in a HEPES lysis buffer (30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, pH 8, 10 mM NaCl, 2 mM Na_3VO_4, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 100 µg/ml 4-(2-aminoethyl)benzenesulfonylfluoride, 6.4 mg/ml Sigma 104 phosphatase substrate), incubated on ice for 30 min and centrifuged at 13,000 x g to clear the lysates. Protein content was determined by the Bradford method. Total cell lysates (30 to 50 µg protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were incubated in blocking solution and probed with primary antibodies overnight. Positive antibody reactions were visualized using peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system (Renaissance, NEN, Boston, MA) according to the manufacturer's instructions. For quantitation of protein expression levels, X-ray films were scanned on a Molecular Dynamics personal SI densitometer and analyzed using the ImageQuant software package (Version 4.1, Molecular Dynamics, Sunnyvale, CA).

Transfections: HeLa cells were transfected with plasmids encoding dominant-negative mutants of Cdk1, Cdk2 and Cdk3 using Lipofectamine PLUS™ in serum-containing medium according to the manufacturer's instructions. Media containing the DNA/lipid complexes was removed after three hours, replaced with complete growth medium and cells were harvested after 48 h. Protein

lysates were prepared and analyzed by SDS-PAGE and Western Blot analysis as described above.

RNA Isolation and Northern Blotting: Total RNA was isolated from HeLa cells using RNeasy Kit (Qiagen, Valencia, CA). RNA concentrations were determined spectrophotometrically using a DU640 Spectrophotometer (Beckman Instruments, Fullerton, CA). Northern blotting was performed using NorthernMax™ system (Ambion, Austin, TX) according to the manufacturer's instructions. Briefly, 5 µg total RNA was separated on 1% denaturing agarose gel containing 2.2 M formaldehyde, transferred to Nytran® SuPerCharge membrane (Schleicher&Schuell), UV crosslinked and processed for detection of mRNA. A 711 base pair, DIG-labeled, anti-sense single strand DNA probe was generated by asymmetric PCR amplification using PCR DIG Probe Synthesis Kit. Briefly, the template for probe synthesis was a 711 bp PCR product at the 3' end of human Cdc25A cDNA; this template was generated by conventional PCR methodology using the following primers: 5'–AAGAGGAGGAAGAGCATGTC–3' (Primer A) and 5'–TCAGAGCTTCTTCAGACGAC–3' (Primer B). The DIG-labeled probe was generated by asymmetric PCR from this template using primer B. Overnight hybridization of the probe to the immobilized RNA was carried out in ULTRAhyb™ Ultrasensitive Hybridization Buffer (Ambion) and the membrane was processed using DIG Wash and Block Buffer Set. The hybridized probe/anti-DIG-AP complex was visualized on X-ray film (Kodak, Rochester, NY) after incubation of the membrane with CDP *Star*. Relative

intensities of the hybridization signals were quantified as described above for Western Blotting.

Results

Exposure of asynchronous HeLa cells to the cdk inhibitor roscovitine (10 μM) resulted in a marked increase in Cdc25A protein levels at 24 hr (Figure 1). In accordance with previously published results (28), roscovitine treatment of HeLa cells resulted in an increase in Cdc25B levels, presumably due to inhibition of Cdk1/Cyclin A-mediated targeting of Cdc25B for proteasomal-mediated degradation (Figure 1). Roscovitine treatment had no effect on Cdc25C protein levels, whose activity is predominantly regulated by cytoplasmic sequestration and inactivation (Figure 1) (30-32). Cdc25A protein levels increased in a concentration- and time-dependent manner (Figures 2 and 3), suggesting that this increase was due to the specificity of roscovitine as a cdk inhibitor; similar results were obtained with olomucine, a cdk inhibitor with a similar selectivity profile but with reduced potency (Figure 2C and data not shown). Because Cdc25A protein levels were elevated rapidly, namely within one hour of roscovitine treatment (Figure 3), it seems unlikely that the increased Cdc25A protein levels were simply due to cell cycle perturbation.

Regulation of Cdc25A protein levels by DNA damage checkpoints has previously been reported to be a p53-independent event; Cdc25A levels are also known to be affected by the high-risk human papillomavirus (HPV) E7 oncoprotein (19, 22, 33). To test whether the increase in Cdc25A protein levels due to cdk inhibition was dependent on p53 or HPV status (HeLa cells are HPV-positive), we treated MCF-7 human mammary adenocarcinoma cells, wild type for p53 and HPV-negative, with increasing concentrations of roscovitine for 24 hr.

As seen in HeLa cells, Cdc25A protein levels in MCF-7 cells increased in a concentration-dependent manner following roscovitine treatment, indicating that increases in Cdc25A levels resulting from cdk inhibition were independent of p53 activity or HPV status (Figure 2B).

Because roscovitine is a broad-spectrum cdk inhibitor, we next determined which cdk was involved in regulating Cdc25A protein levels. HeLa cells were transfected with dominant-negative mutants of Cdk1, Cdk2 and Cdk3, as these are the predominant roscovitine-sensitive cdks in HeLa cells. Only genetic inhibition of Cdk2 kinase activity resulted in increased Cdc25A protein levels; genetic inhibition of a Cdk1 or Cdk3 had no effect on Cdc25A levels (Figure 4). We observed no significant alteration in the HeLa cell cycle profile 48 h after transfection with the dominant-negative Cdk2 mutant, consistent with the recent report by Tetsu and McCormick (34). Thus, the increase in Cdc25A protein levels after ectopic expression of the dominant-negative mutant of Cdk2 was not secondary to cell cycle arrest. These results indicate that Cdk2 kinase activity plays an important role in regulating Cdc25A protein levels in asynchronous cells.

Since Cdc25A expression can be regulated at both the transcriptional and post-translational levels, we next investigated the mechanism responsible for increases in Cdc25A levels following inhibition of Cdk2 activity. In response to genetic insults or inhibition of DNA synthesis, Cdc25A is phosphorylated and targeted for rapid ubiquitin-mediated proteolytic degradation by the checkpoint kinases chk1 and cds1/chk2 (11, 21, 22). In addition, transcription from the Cdc25A promoter can be activated by E2F and c-myc transcription factors (17-

19). Because Cdc25A has been reported to be a highly labile protein in interphase and an increase in the half-life of a labile protein would result in a significant accumulation of that protein, we explored whether inhibition of Cdk2 kinase activity altered the half-life of Cdc25A in asynchronous cells. Following a 24 hr treatment with roscovitine or vehicle control, HeLa cells were treated with 10 μg/ml cycloheximide for 0 to 60 min and Cdc25A levels were examined by Western blotting. The basal half-life of Cdc25A was 6.26 ± 0.78 min, which is in agreement with previous reports (11, 20). Roscovitine-mediated inhibition of Cdk2 kinase activity doubled the half-life of Cdc25A (Figure 5), which readily could account for the observed time-dependent increases in Cdc25A protein levels. To confirm that the increased Cdc25A protein levels were not affected by a transcriptionally-mediated mechanism, Cdc25A mRNA levels were examined by Northern blotting. Roscovitine treatment of HeLa cells did not significantly increase Cdc25A mRNA levels, confirming that Cdk2 kinase activity affects Cdc25A protein levels by a post-transcriptional mechanism (Figure 6). These results were independently confirmed by RT-PCR (data not shown).

Discussion

Cdc25A biology is undergoing a paradigm shift, drifting away from its narrow role as critical regulator of the G1/S checkpoint to a more broad responsibility in the cell cycle with an essential function in mitosis. Specifically, it is now known that Cdc25A levels are at their highest during late G2/M and that degradation of Cdc25A is necessary for the G2/M checkpoint in response to DNA damage (11, 12). The original models describing the regulation of Cdc25A are being refined to include recent data thoroughly detailing protein stability as one of its key regulatory mechanisms (11, 12, 20-23). The relationship between Cdc25A and Cdk2 was originally that of an autoamplification feedback loop where Cdk2 contributed to the activation of Cdc25A and Cdc25A contributed to the activation of Cdk2 to amplify the activities of both proteins to a high enough level to enable progression through the G1/S transition (9). Here we report that Cdk2 kinase activity contributes to the labile nature of Cdc25A in interphase, and this kinase activity may in fact be the same Cdk2 kinase activity originally reported to activate Cdc25A. Our results contribute to the understanding of this Cdc25A-cdk feedback loop and support a mathematical model that suggests hyperphosphorylation of Cdc25A by Cdk2 may contribute to its degradation (9, 35). By directly linking Cdc25A stability to the activity of its substrates, physiologic levels of Cdc25A can be maintained in a tight feedback loop to prevent catastrophic deregulated Cdc25A protein levels or activity. This relationship between increased activity and decreased protein stability has been described for another protein phosphatase, PTEN. PTEN phosphorylation

maintains the protein in a stabilized state with decreased phosphatase activity; upon loss of phosphorylation in the C-terminal PTEN tail, catalytic activity is increased and protein stability is decreased (36). Although our data does not specifically detail the nature of the Cdk2/Cyclin complex that contributes to the inherent instability of Cdc25A in interphase or the detailed molecular mechanism involved, there are several possible testable hypotheses. It has recently been reported that Cdc25A can associate with elements of the SCF ubiquitin ligases and may be a target of the APC^{cdh1} and SCF ubiquitin ligases (20). However, it remains uncertain how Cdc25A might be targeted to these ubiquitin ligases. Other cell cycle regulatory proteins, notably p27 and Cyclin E, are targeted by the SCF ubiquitin ligase for proteolytic degradation by a phosphorylation-dependent mechanism, while conversely, phosphorylation may not be necessary for p21 and Cyclin D degradation mediated by SCF ligases (37, 38). It remains unclear whether phosphorylation of Cdc25A is a necessary event preceding ubiquitin ligase association, as Cdc25A is phosphorylated prior to its degradation in response to genetic insults and is rescued from proteolytic degradation in mitosis by Cdk1/Cyclin B-mediated phosphorylation (12). However, our results support a role for Cdk2-mediated phosphorylation of either Cdc25A itself or a specific effector protein(s) necessary for the rapid degradation of Cdc25A. While our results cannot rule out the involvement of Chk1 and Cds1/Chk2 as downstream effectors of the Cdk2 kinase activity responsible for Cdc25A degradation, their role may be unique to proteasomal targeting of Cdc25A following cellular stress and may not play a role in regulating Cdc25A levels in the absence of cellular

stress. A similar model seems to regulate Cdc25C, which is inactivated in the G2/M checkpoint by checkpoint kinase(s)-dependent phosphorylation, 14-3-3 association and cytoplasmic sequestration; Cdc25C, however, is maintained inactive and sequestered in the cytoplasm during interphase in the absence of cellular stress by Cdc twenty-five C associated protein kinase (C-TAK1) (39).

Based on our results and the above mentioned studies, we propose the following model: Cdc25A levels are upregulated by transcription in late G1 to a level that enables Cdk2/Cyclin E activation to promote transition from G1 into S phase. Then Cdc25A levels are carefully controlled via Cdk2/Cyclin E and Cdk2/Cyclin A kinase activity through S phase and into late G2 phase. Once Cdk1/Cyclin B is activated Cdc25A protein levels are released from this strict regulatory loop and permitted to increase as the cells approach the G2/M transition and reach their maximal levels, which are required for mitosis (11, 12).

As deregulated cell cycle progression is one of the hallmarks of cancer, regulation of cell cycle proteins has taken a prominent position in efforts to design new therapeutic approaches, including inhibitors of cdks (13). While rational drug discovery efforts are laudable, our results provide a cautionary note. They suggest that inhibitors of the catalytic activity of Cdk2 may have the unexpected consequence of elevating the expression of the proto-oncogene Cdc25A.

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Footnotes

[1]Abbreviations: CHX, cycloheximide; DN, dominant negative; AP, alkaline phosphatase; Cdk, cyclin-dependent kinase; DIG, digoxigenin; HPV, human papillomavirus.

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Figure Legends

Figure 1: Roscovitine Treatment Increases Cdc25A Levels. HeLa cells were treated for 24 hr with vehicle control (DMSO) or 10 μM roscovitine. Cdc25A, Cdc25B, Cdc25C, and β-tubulin (loading control) levels were examined by Western blot.

Figure 2: Roscovitine Treatment Increases Cdc25A Levels in a Concentration-Dependent Manner. HeLa (Panels A & C) and MCF-7 (Panel B) cells were treated for 24 hr with vehicle control (DMSO) or increasing concentrations of roscovitine (Panels A & B) or olomucine (Panel C). Cdc25A and β-tubulin (loading control) levels were examined by Western blot. Cdc25A levels are expressed as fold increase over vehicle control ± S.E.M (n = 3-5) (panels A & B). Increasing concentrations of roscovitine significantly increased Cdc25A levels (ANOVA $p < 0.05$).

Figure 3: Roscovitine Treatment Increases Cdc25A Levels in a Time-Dependent Manner. HeLa cells were treated with 10 μM roscovitine for 0-2 hr. Cdc25A and β-tubulin (loading control) levels were examined by Western blot (Panel A). Cdc25A levels from roscovitine treated cells are expressed as fold increase over control (0 hr) ± S.E.M (n = 5 to 7) (Panel B). Cdc25A levels significantly increase with increasing length of roscovitine treatment (ANOVA $p < 0.05$).

Figure 4: Dominant-Negative (DN) Cdk2 Expression Increases Cdc25A Levels in HeLa Cells. HeLa cells were transfected with pcDNA3.1 (vector control) or vectors encoding DN Cdk1, DN Cdk2, or DN Cdk3. 48 hr after transfection, Cdc25A, Cdk1, Cdk2, Cdk3 and β-tubulin (loading control) levels were examined by Western blot (Panel A). Cdc25A levels are expressed as fold increase over vector control ± S.E.M (n = 3) (Panel B). DN Cdk2 expression significantly increases Cdc25A levels (ANOVA $p < 0.05$).

Figure 5: Inhibition of Cdk2 Activity Increases Cdc25A Half-Life in Asynchronous Cells. HeLa cells were treated for 24 hr with vehicle control (DMSO) or 10 μM roscovitine followed by 10 μg/ml cycloheximide (CHX) for 0-60 min. Cdc25A and vinculin (loading control) levels were examined by Western blot (Panel A). Cdc25A levels are expressed as percent of control ± S.E.M (n = 3) (Panel B). Roscovitine treatment significantly increased the half-life of Cdc25A (Student's t-test $p < 0.05$).

Figure 6: Roscovitine Treatment does not Increase Cdc25A mRNA Levels. HeLa cells were treated for 24 hr with vehicle control (DMSO) or 10 μM roscovitine. Total RNA was extracted as described in Materials and Methods and Cdc25A and β-actin levels were examined by Northern Blot (Panel A). Cdc25A mRNA levels are expressed as arbitrary units ± S.E.M (n = 3) (Panel B). Roscovitine treatment does not significantly increase Cdc25A mRNA levels.

Figure 1

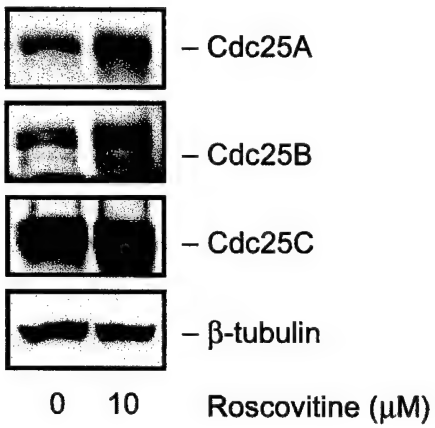
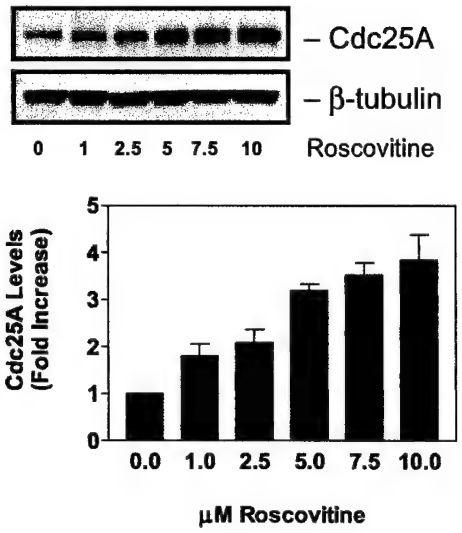
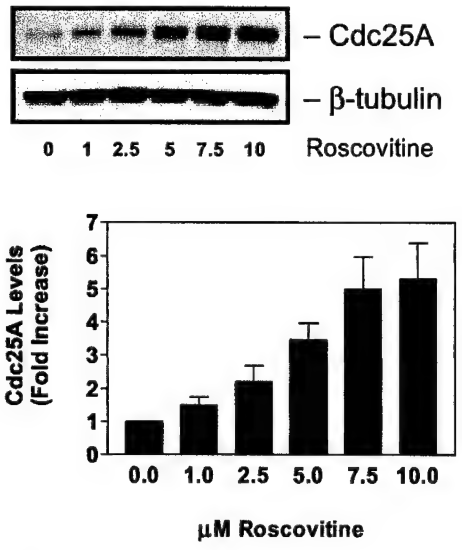


Figure 2

A.


– Cdc25A
– β-tubulin
0 1 2.5 5 7.5 10
Roscovitine
Cdc25A Levels (Fold Increase)
0 1 2 3 4 5
0.0 1.0 2.5 5.0 7.5 10.0
μM Roscovitine

B.


– Cdc25A
– β-tubulin
0 1 2.5 5 7.5 10
Roscovitine
Cdc25A Levels (Fold Increase)
0 1 2 3 4 5 6 7
0.0 1.0 2.5 5.0 7.5 10.0
μM Roscovitine

C.

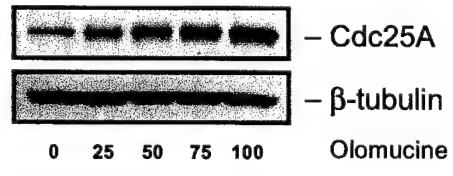
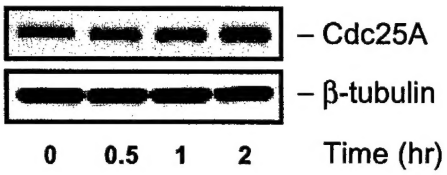

– Cdc25A
– β-tubulin
0 25 50 75 100
Olomucine

Figure 3

A.


– Cdc25A
– β-tubulin
0 0.5 1 2
Time (hr)

B.

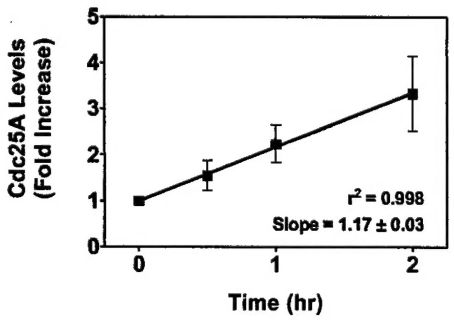
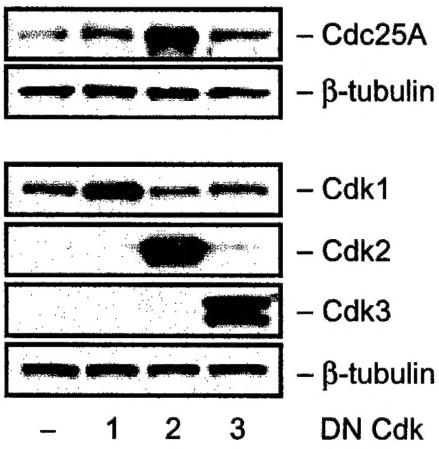

Cdc25A Levels (Fold Increase)
5
4
3
2
1
0
0
1
2
Time (hr)
r² = 0.998
Slope = 1.17 ± 0.03

Figure 4

A.


– Cdc25A
– β-tubulin
– Cdk1
– Cdk2
– Cdk3
– β-tubulin
– 1 2 3 DN Cdk

B.

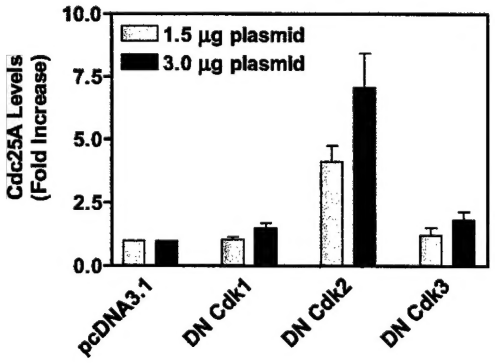
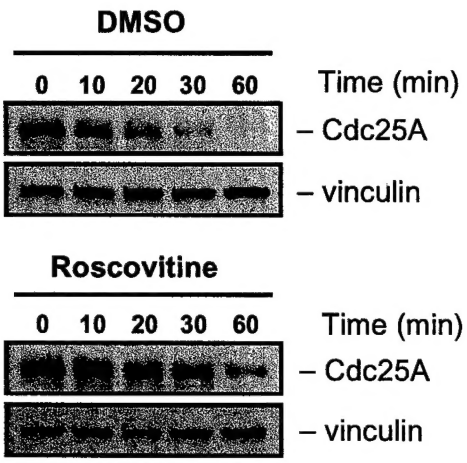

Cdc25A Levels (Fold Increase)
10.0
7.5
5.0
2.5
0.0
1.5 µg plasmid
3.0 µg plasmid
pcDNA3.1
DN Cdk1
DN Cdk2
DN Cdk3

Figure 5

A.


DMSO
0 10 20 30 60
Time (min)
– Cdc25A
– vinculin
Roscovitine
0 10 20 30 60
Time (min)
– Cdc25A
– vinculin

B.

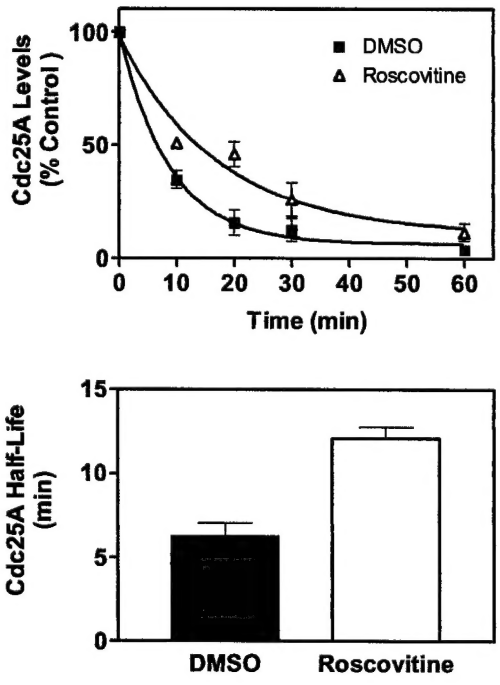
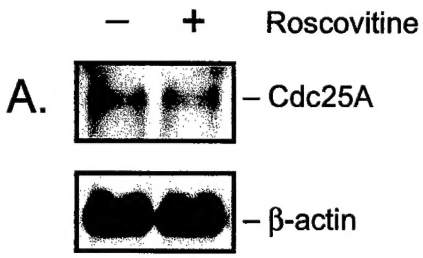
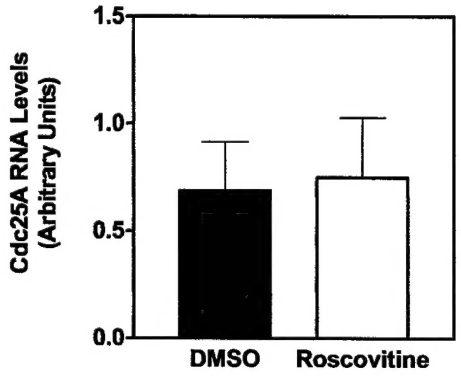

Cdc25A Levels (% Control)
100
50
0
DMSO
Roscovitine
0 10 20 30 40 50 60
Time (min)
Cdc25A Half-Life (min)
15
10
5
0
DMSO
Roscovitine

Figure 6


– +
Roscovitine
A.
– Cdc25A
– β-actin

B.


Cdc25A RNA Levels
(Arbitrary Units)
1.5
1.0
0.5
0.0
DMSO
Roscovitine